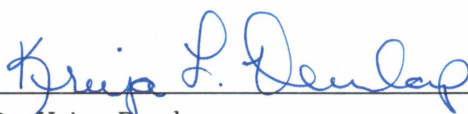


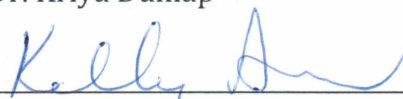
EFFECTS OF METHYLMERCURY EXPOSURE ON 3T3-L1 ADIPOCYTES

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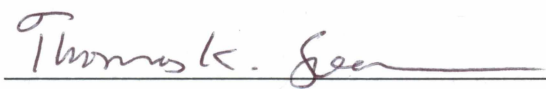
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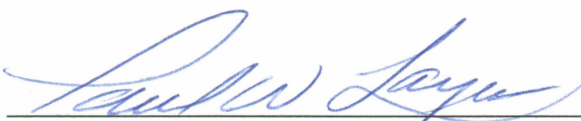

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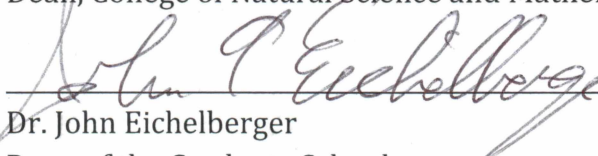

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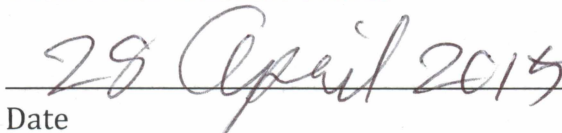

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EFFECTS OF METHYLMERCURY EXPOSURE ON 3T3-L1 ADIPOCYTES

A
THESIS

Presented to the Faculty
of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

By

Theresa Vertigan, B.A.

Fairbanks, Alaska

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Abstract

Mercury-containing compounds are environmental pollutants that have become increasingly consequential in the Arctic regions of North America due to processes of climate change increasing their release and availability at northern latitudes. Currently, the form of mercury known to be most detrimental to human health is methylmercury, CH_3Hg^+ , which is found in the environment primarily accumulated in the tissues of predatory fish, including those consumed by Alaska Natives through subsistence gathering. Much is known about the neurotoxicity of methylmercury after exposure to high concentrations, but little is known about toxicity to other tissues and cell types, particularly for long-term exposure and the lower concentrations that would occur through fish consumption. This study aims to investigate the potential effects of methylmercury exposure on adipocytes, the main cellular components of adipose (fat) tissue, and explore possible consequences of exposure on metabolic disorders such as obesity and diabetes.

Effects of methylmercury exposure on 3T3-L1 adipocytes in culture were assessed using assays for cytotoxicity and an ELISA assay for vascular endothelial growth factor (VEGF), a signaling molecule shown to be important for maintaining metabolic status in adipose tissue. Results showed that exposure to methylmercury leads to significant toxicity in adipocytes at exposures of 100 ng/mL during later stages of differentiation, but lower methylmercury concentrations produced little to no toxicity. Results also indicate that VEGF secretion is elevated in adipocytes exposed to methylmercury after the process of differentiating into mature, fat-storing cells. These results provide a basis for further exploration into metabolic consequences of methylmercury exposure.

Table of Contents

Signature Page.....	i
Title Page.....	iii
Abstract.....	v
Table of Contents	vii
List of Figures	xi
List of Tables	xiii
List of Abbreviations	xv
Acknowledgements.....	xvi
Chapter 1: Introduction and Background	1
1.1 Mercury	1
1.1.1 Methylmercury	2
1.1.2 Biochemistry of Methylmercury	2
1.2 Adipose tissue and obesity	4
1.2.1 Adipokines	5
1.2.2 VEGF	7
1.2.3 3T3-L1 adipocytes.....	9
1.3 Significance and research hypotheses	11
1.3.1 Significance.....	11
1.3.2 Research hypotheses.....	12

Chapter 2: Materials and Methods	13
2.1 Culture of 3T3-L1 pre-adipocytes.....	13
2.2 LDH assay	17
2.3 VEGF ELISA.....	19
2.4 Statistical Analysis.....	21
Chapter 3: Results.....	23
3.1 Morphological changes to 3T3-L1 cells upon differentiation and MeHg exposure	23
3.1.1 Changes to cells during differentiation	23
3.1.2 Changes to cells after exposure to MeHg during differentiation	23
3.1.3 Changes to cells after exposure to MeHg without differentiation	28
3.1.4 Changes to cells after exposure to MeHg post-differentiation.....	29
3.2 Cytotoxic effects of MeHg exposure on 3T3-L1 cells.....	30
3.2.1 Cytotoxic effects on pre-adipocytes during differentiation	31
3.2.2 Cytotoxic effects on non-differentiating pre-adipocytes.....	33
3.2.3 Cytotoxic effects on mature adipocytes	34
3.2.4 Cytotoxic effects on non-differentiated cells after 12 days of no exposure	35
3.3 Effects of MeHg exposure on VEGF secretion from 3T3-L1 cells	36
3.3.1 Effects on VEGF secretion from pre-adipocytes during differentiation.....	36
3.3.2 Effects on VEGF secretion from non-differentiating pre-adipocytes	38
3.3.3 Effects on VEGF secretion from mature adipocytes	39
3.3.4 Effects on VEGF secretion from non-differentiated cells after 14 days of no exposure.....	41

Chapter 4: Discussion and Future Directions.....	42
4.1 Discussion	42
4.1.1 Significance of overall results.....	42
4.1.2 Concentration-dependent cytotoxicity of MeHg.....	43
4.1.3 Concentration-dependent effects of MeHg on secretion of VEGF.....	44
4.1.4 Physiological relevance of results	47
4.2 Future Directions	48
4.2.1 Effects of MeHg exposure on secretion of other adipokines.....	48
4.2.2 Cytotoxic effects after brief MeHg exposure	49
4.2.3 Destination of MeHg within the cell	49
4.2.4 Confounding effects of omega-3 fatty acids on toxicity of MeHg.....	50
References.....	52

List of Figures

Figure 1.1: Adipose tissue secretes a variety of adipokines.....	6
Figure 1.2: Adipokine signaling is dependent on metabolic status.....	7
Figure 1.3: Adipocyte differentiation	10
Figure 2.1: 3T3-L1 cell populations/MeHg exposure schemes	14
Figure 2.2: Schematic of generic sandwich ELISA.....	19
Figure 3.1: Healthy confluent 3T3-L1 cells.....	24
Figure 3.2: Differentiating 3T3-L1 cells exposed to 200 ng/mL MeHg.....	25
Figure 3.3: Differentiating 3T3-L1 cells exposed to 100 ng/mL MeHg.....	27
Figure 3.4: Differentiating 3T3-L1 cells exposed to 25 ng/mL MeHg.....	28
Figure 3.5: Non-differentiating 3T3-L1 cells exposed to 200 ng/mL MeHg	29
Figure 3.6: Mature 3T3-L1 adipocytes exposed to 200 ng/mL MeHg.....	30
Figure 3.7: Cytotoxicity of MeHg in differentiating 3T3-L1 cells	32

Figure 3.8: Cytotoxicity of MeHg in non-differentiating 3T3-L1 cells.....	33
Figure 3.9: Cytotoxicity of MeHg in mature 3T3-L1 adipocytes.....	35
Figure 3.10: Cytotoxicity in non-differentiated cells exposed to MeHg for 3 days	36
Figure 3.11: Effects of MeHg on VEGF secretion from differentiating cells	37
Figure 3.12: Effects of MeHg on VEGF secretion from non- differentiating cells	39
Figure 3.13: Effects of 3 days of MeHg exposure on VEGF secretion from mature adipocytes.....	40
Figure 3.14: Effects of 3 days of MeHg exposure on VEGF secretion from non-differentiated cells.....	41

List of Tables

Table 2.1: Media and chemical components for 3T3-L1 cell culture.....	15
Table 2.2: 3T3-L1 cell differentiation protocol.....	16
Table 2.3: Volumes of MeHg added to cell culture media	17

List of Abbreviations

ATCC	American Type Culture Collection
cAMP	Cyclic adenosine monophosphate
DMEM	Dulbecco's Modified Eagle Medium
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
IBMX	Methylisobutylxanthine
IL-6	Interleukin-6
LDH	Lactate dehydrogenase
MeHg	Methylmercury
ng/mL	Nanograms per milliliter
pg/mL	Picograms per milliliter
ppb	Parts per billion
ppm	Parts per million
rpm	Revolutions per minute
RT	Room temperature
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor

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Chapter 1: Introduction and Background

1.1 Mercury

Mercury is an element that exists naturally within the Earth's crust and is constantly cycled into and out of different locations in the environment, due to both natural processes and human activities (Arctic Monitoring and Assessment Program, 2011; Krabbenhoft & Sunderland, 2013). Industrial processes, including the burning of coal and production of metals, are the main contributors to anthropogenic mercury release into the atmosphere, with an estimated 65% of air emissions originating in Asian countries, primarily China (Arctic Monitoring and Assessment Program, 2011). Due to atmospheric circulation trends, large amounts of mercury are deposited into terrestrial and aquatic environments at Arctic latitudes, a process which may be exacerbated by the increasing effects of global climate change (Fisher et al., 2012; Krabbenhoft & Sunderland, 2013; Stern et al., 2012).

Several species of mercury exist within the environment, and may interconvert through reactions catalyzed either chemically (in the environment) or biologically (within organisms). Atmospheric mercury released through industrial processes generally exists in its elemental form, Hg^0 (Arctic Monitoring and Assessment Program, 2011; Fisher et al., 2012; Krabbenhoft & Sunderland, 2013; Stern et al., 2012), while ionized inorganic mercury (Hg^{2+}) and so-called "organic" mercury (e.g. methylmercury, HgCH_3^+) are the forms most often encountered in the human environment. The latter is typically formed through methylation of inorganic mercury by microorganisms living in aquatic and wetland environments (Krabbenhoft & Sunderland, 2013).

1.1.1 Methylmercury

Methylmercury (MeHg) is unique among the mercury species in its ability to bioaccumulate and biomagnify within aquatic food webs. Predatory fish, which tend to occupy higher trophic levels within these systems, often contain MeHg at concentrations thousands of times higher than those found in the surrounding environment. Consumption of predatory fish as food thus represents the most common source of MeHg exposure both to humans and to wildlife (Krabbenhoft & Sunderland, 2013). Given the increased concentrations of mercury in the environment at northern latitudes, exposure to MeHg is therefore of great concern to native communities, including Alaska Natives, who rely on predatory fish as a staple food in their subsistence-based diets (Lemire et al., 2014; Mahaffey, 1999).

1.1.2 Biochemistry of Methylmercury

Much of the current knowledge of MeHg's detrimental health effects has arisen from observation of accidental catastrophic release of MeHg into the environment, such as in Minamata, Japan in the 1960s (Kanda et al., 2014; Mahaffey, 1999). Observation of patients following such incidents has shown that acute exposure to high concentrations of MeHg can cause severe negative impacts on the brain and nervous system, causing damage both in exposed adults and in fetuses exposed *in utero*. MeHg, unlike inorganic ionic mercury species, is capable of easily crossing the human placenta, which in part has led to increased concern about MeHg exposure to the developing embryo and fetus (Mahaffey 1999). Effects

of more chronic low-level MeHg exposure are less well understood, but neurodevelopmental and neurological damage in general appear to be associated with such exposure (Weiss, 2007).

The MeHg molecule itself has a high affinity for thiol groups, and is able to covalently associate with accessible thiol groups on proteins, particularly those found in cysteine residues. Such modification is known as “S-mercuration”, and is thought to provide the basis for the cytotoxic and neurotoxic effects of MeHg *in vivo*, though the precise biochemical mechanisms by which this occurs are still not entirely clear (Kanda et al., 2014). One known consequence of S-mercuration is that free cysteine molecules that have been covalently modified by MeHg may be recognized as methionine molecules by amino acid transporters in the brain, and as such are able to cross the blood-brain barrier (Kerper, Nazzareno, & Clarkson, 1992). More recent studies have suggested that S-mercuration of particular proteins within the cytoprotective Keap1/Nrf2 pathway may be responsible for some of the toxic effects of MeHg (Kumagai et al., 2013), while still other research shows that exposure to MeHg may upregulate growth factor pathways in neuronal cells in culture (Hirooka et al., 2013).

While considerable focus has been directed toward effects of MeHg exposure in the brain and in cultured neurons, less research exists examining possible effects on other organs or cell types. Epidemiological studies have examined possible effects of MeHg on diabetes and metabolic dysfunction, and while some results have indicated a correlation between MeHg and eventual development of diabetes (He et al., 2013), others have shown no significant correlation between exposure to MeHg and diabetes in patients living in a MeHg-contaminated area (Futatsuka, Kitano, & Wakamiya, 1996). A study by Yamamoto et

al. (2013) examined interactions between diabetes and MeHg toxicity, concluding that diabetic animals experienced more pronounced toxic effects from MeHg than metabolically normal animals, and providing evidence of a role for MeHg in metabolic dysfunction.

Studies by Barnes, Hanlon and Kircher (2003) and Barnes and Kircher (2005) provide the only results to date on the effects of exposure of adipocytes to mercury compounds, though the mercury species used in these studies was inorganic mercury (Hg^{2+}), which is less relevant to human health than MeHg, for reasons mentioned above. Regardless, their studies showed a significant effect on the ability of pre-adipocytes in culture to differentiate into mature adipocytes, as well as some effect on insulin signaling in these cells, which again provides a basis for further exploration of mercury species as potential modulators of metabolic processes in adipocytes.

1.2 Adipose tissue and obesity

Obesity in humans is a condition associated with an imbalance between energy intake and energy expenditure, where excess energy consumed is stored mainly in adipose tissue in the form of lipid (Goossens, 2008). Specifically, lipid is stored in specialized cells called adipocytes, which store the lipid within vesicles that increase in size as more lipid is deposited there. Excessive storage of lipid leads to an increase in adipocyte size, which then leads to an increase in overall size of adipose tissue deposits (Bays et al., 2008).

Obesity in humans is associated with significant dysfunction in overall metabolic processes and an increase in adiposity has been linked with type 2 diabetes (Bays et al., 2008, Goossens, 2008; Kahn, Hull, & Utzschneider, 2006; Murdolo & Smith, 2006). Type 2 diabetes involves dysfunctional insulin signaling and sensitivity, and numerous studies

have sought to establish the specific mechanisms for development of this disease as well as its relationship to obesity. Studies have provided considerable evidence associating type 2 diabetes with increases in mitochondrial dysfunction, chronic low-level inflammation, and hypoxic conditions in adipose tissue (Bakhai, 2008; Bhargava & Lee, 2012; Bays et al., 2008; Goossens, 2008; Kahn et al., 2006; Murdolo & Smith, 2006; Trayhurn, Bing, & Wood, 2005). The specific cellular mechanisms underlying these associations generally involved altered cell signaling pathways within diabetic and obese individuals, particularly involving adipose tissue and adipocytes.

Adipocytes initially develop from progenitor cells called pre-adipocytes, which are induced to differentiate into mature adipocytes through a process known as adipogenesis, initiated by signaling cascades involving growth and transcription factors (Gregoire, Smas, & Sul, 1998; Rosen & MacDougald, 2006; Stephens, 2012). Adipogenesis is associated with a change in cell morphology and appearance of intracellular lipid droplets. Though specifics of adipocyte turnover within adipose tissue are still an active area of research, current estimates suggest a cellular turnover rate of approximately 10% annually, defined as 10% of adipocytes in a population transformed from pre-adipocytes in a given year (Arner et al., 2010; Rigamonti et al., 2011; Spalding et al., 2008).

1.2.1 Adipokines

In the past, adipose tissue was considered little more than a location for passive storage of energy. Recent evidence, however, has shown it to be capable of directly influencing signaling pathways involved in energy homeostasis, angiogenesis, and insulin sensitivity (Figure 1.2). This is primarily achieved through secretion and recognition of protein molecules known as adipokines (Bakhai, 2008).

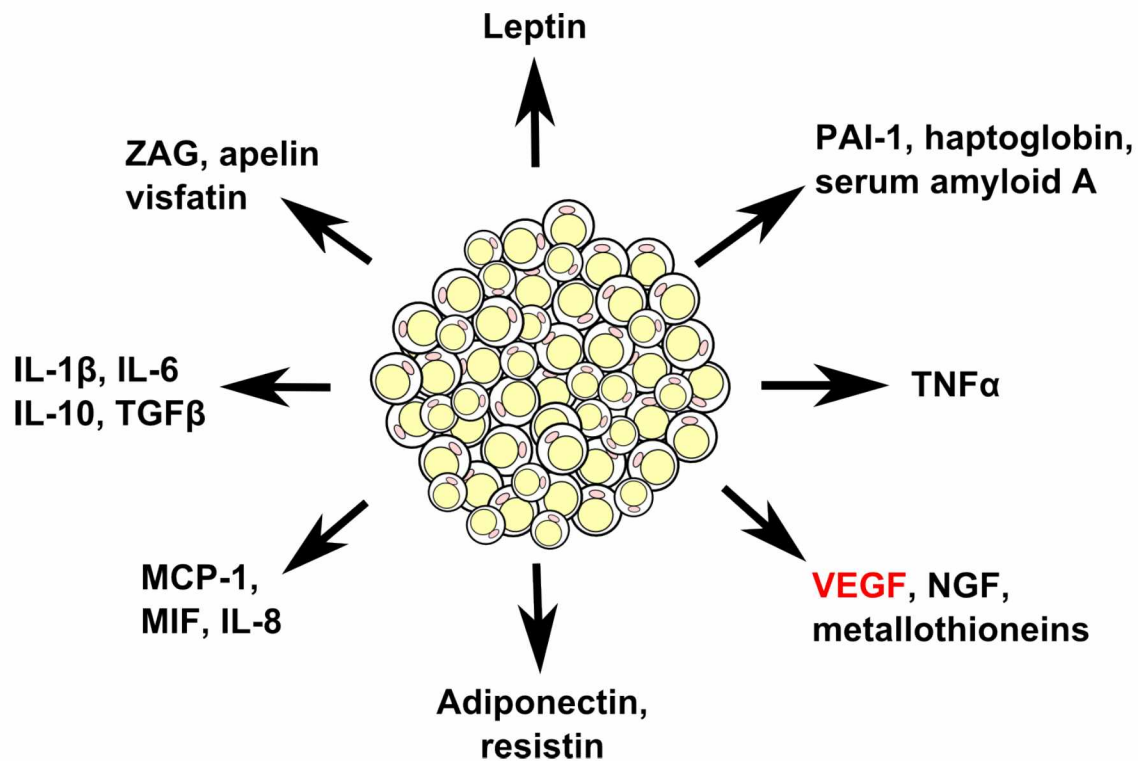


Figure 1.1: Adipose tissue secretes a variety of adipokines (adapted from Trayhurn et al., 2005)

Some adipokines are produced solely by adipose tissue (e.g. leptin and adiponectin), while many others are also used by other tissues and organs. Examples of the latter include several pro-inflammatory molecules like tumor necrosis factor (TNF) and interleukin 6 (IL-6), growth factors like vascular endothelial growth factor (VEGF), and cytoprotective proteins like metallothioneins (Bakhai, 2008; Trayhurn et al., 2005). The ability of adipokines to directly influence signaling pathways relevant to energy balance and metabolism suggests that they may play important roles in processes leading to diabetes and obesity (Figure 1.2), and indeed adipokine dysregulation has been observed in models of these diseases (Bakhai, 2008; Bays et al., 2008; Trayhurn et al., 2005; Zou & Shao, 2008). An example of a specific adipokine, VEGF, and evidence for its potential role in metabolic dysfunction, are described below.

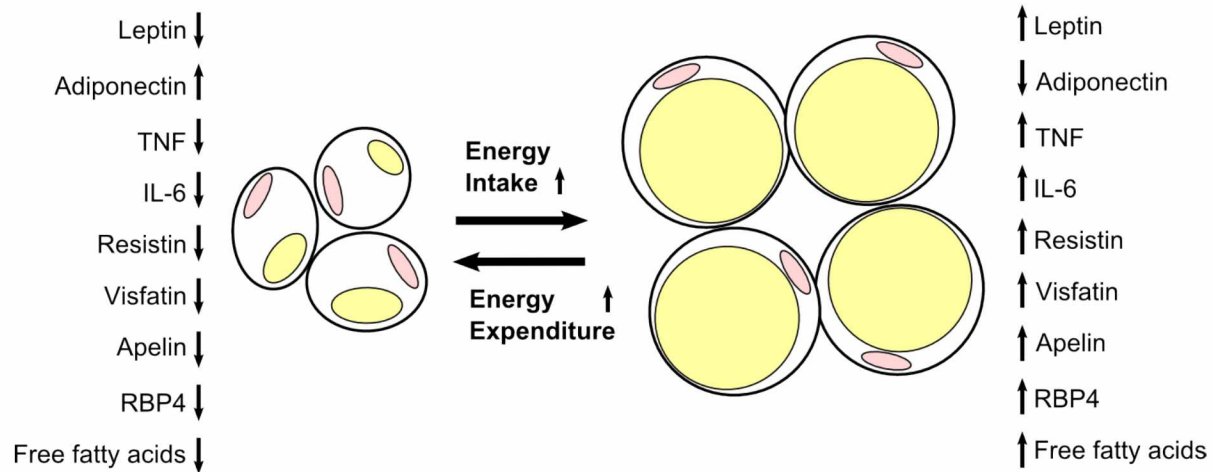


Figure 1.2: Adipokine signaling is dependent on metabolic status (adapted from Zou & Shao, 2008)

1.2.2 VEGF

The family of proteins called vascular endothelial growth factors (VEGF) are signaling molecules that are critically important in the promotion of angiogenesis, or formation of new blood vasculature in tissue. Up to seven different members of the VEGF family are currently recognized, with VEGF-A the best characterized and most recognized for its importance in angiogenic processes involving endothelial cells (Takahashi & Shibuya, 2005).

VEGF-A (often referred to simply as VEGF) is a soluble homodimeric glycoprotein that binds with high affinity to its target receptors, known as the VEGF receptors (VEGFR-1 and VEGFR-2), which exist mainly as cell-surface receptors (Ferrara, 2004; Takahashi & Shibuya, 2005). The binding of VEGF to and subsequent activation of these receptors instigates phosphorylation-based signaling cascades within the target cell, leading to upregulated transcription of genes involved in angiogenesis (Ferrara, 2004). A primary

purpose of angiogenesis is to vascularize tissues and organs, ensuring sufficient flow of nutrients and oxygen into those regions; increased VEGF signaling thus often occurs in response to hypoxic conditions, including in abnormal tissues such as tumors (Ferrara, 2004). Indeed, much of the current knowledge of mechanisms of VEGF signaling have arisen from research on cancer, in which increased VEGF signaling correlates to increased vasculature of a tumor, which often correlates to poorer prognosis for the patient (Ferrara, 2004; Takahashi & Shibuya, 2005).

The proper functioning of adipose tissue may also be sensitive to changes in vascularization, and so VEGF in recent years has begun to be studied as a molecule of interest in conditions affecting cell signaling processes in adipose tissue and adipocytes, including diabetes and obesity (Cao, 2013; Cullberg et al., 2013; Disanzo & You, 2014; Elias, Franckhauser, & Bosch, 2013; Hagberg et al., 2012; Honek et al., 2014; Loebig et al., 2010; Schlich et al., 2013; Sun et al., 2012; Trayhurn, Wang, & Wood, 2008). So far, however, the consequences of increased VEGF expression in adipose tissue and its possible connection to metabolic dysfunction remain somewhat mysterious. Some research has indicated that increased VEGF (and thus increased vascularization) in adipose tissue might be beneficial for the metabolic status and/or insulin sensitivity of the host, since vascularization tends to prevent hypoxic conditions, and some negative effects of obesity and metabolic disease may be due to hypoxia in adipose tissue (Elias et al., 2013; Sun et al., 2012; Yilmaz & Hotamisligil, 2013). On the other hand, other studies have shown that increased VEGF/vascularization may be detrimental to adipose tissue in that increased accessibility of nutrients to adipose tissue may contribute to its ability to expand and exacerbate the processes of obesity and metabolic disease (Aprahamian, 2013; Sun et al., 2012; Yilmaz &

Hotamisligil, 2013). In either case, VEGF appears to be directly linked to metabolic status and signaling pathways in adipose tissue, and as such, is worthy of further study to determine its possible role in metabolic disease.

1.2.3 3T3-L1 adipocytes

3T3-L1 cells are a line of adherent pre-adipocyte mouse fibroblasts that is widely used as a model for studying adipocytes, and adipogenesis as observed in these cells is accepted in the research community as a faithful representation of human adipogenesis (Williams, 1997; Martini et al., 2014). As pre-adipocytes, the cells in this line are genetically “committed” to transformation into adipocytes, but have not yet received the chemical signals necessary to differentiate them into mature cells (Figure 1.3).

When exposed to appropriate chemical signals in culture (methylisobutylxanthine [IBMX], dexamethasone, insulin), 3T3-L1 cells are able to differentiate into mature adipocytes (Gregoire et al., 1998; Vishwanath et al., 2013). IBMX is a compound that increases production of intracellular cyclic AMP (cAMP) and inhibits phosphodiesterase, while dexamethasone is a glucocorticoid required for differentiation of 3T3-L1 cells in particular. Both of these compounds appear to upregulate expression of PPAR- γ , a protein known to be critical in the differentiation process. Addition of insulin along with these two compounds has the effect of making accumulation of lipid more efficient (Gregoire et al., 1998). As the cells differentiate, they adopt a noticeably rounded phenotype and begin to

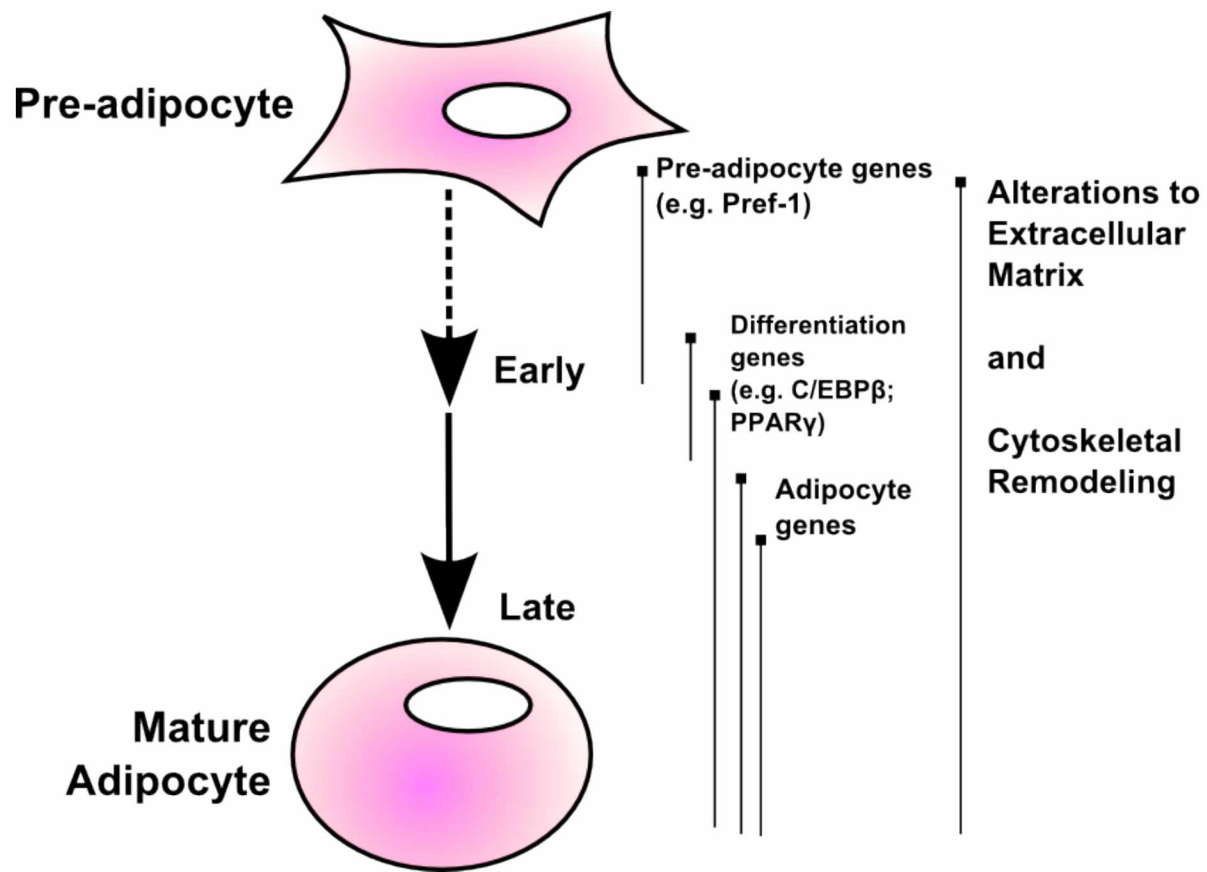


Figure 1.3: Adipocyte differentiation. Changes in gene expression and morphology occur in the transition from pre-adipocyte to mature adipocyte. Adapted from Gregoire et al., 1998

accumulate lipids in vesicles/droplets that become visible as they increase in size; cells are generally considered mature approximately 8-15 days after initiation of differentiation, or when lipid droplets are visible throughout the majority of the cells.

1.3 Significance and research hypotheses

1.3.1 Significance

Mercury-containing compounds are environmental pollutants that have become increasingly consequential in the Arctic regions of North America due to processes of climate change increasing their release and availability at northern latitudes. MeHg is the mercury species considered most detrimental to human health, and is found in the environment primarily accumulated in the tissues of predatory fish, including those consumed by Alaska Natives through subsistence farming. Obesity rates are rising among Alaska Native populations (Makhoul et al., 2010), and as the amount of mercury found in the environment in Arctic regions has also been rising, this project aims to explore whether chronic low-level MeHg exposure may have an effect on physiological processes that contribute to the development of obesity and/or diabetes. Specifically, we aim to investigate the potential effects of MeHg exposure on adipocytes, the main cellular components of adipose tissue.

While the effects of acute MeHg exposure have been observed, and primarily involve acute damage to neurological systems, the long-term effects of chronic low-level exposure to MeHg, such as would be acquired through consumption of mercury-tainted fish, have not yet been fully characterized. Additionally, no studies to date have investigated the effects of MeHg on adipocytes at either acute or chronic exposures. Given the important role of adipose tissue and adipokines in metabolic homeostasis, any effects of exposure to MeHg on adipocytes involving cellular toxicity or altered adipokine signaling could have

implications for a possible role of MeHg in the development or exacerbation of metabolic dysfunctions like obesity and diabetes.

1.3.2 Research hypotheses

Since no literature currently exists examining the effects of low-level MeHg exposure on adipocytes, hypotheses developed for this study were largely exploratory in nature. Specific hypotheses were:

- Exposure to MeHg increases secretion of VEGF by pre-adipocytes (3T3-L1 cell line) in culture in a concentration-dependent manner
- Exposure to MeHg causes increased cytotoxicity in adipocytes in culture (3T3-L1 cell line) in a concentration-dependent manner, leading to reduced VEGF in media.
- Exposure to MeHg interferes with transition of cells from pre-adipocytes to mature adipocytes

Chapter 2: Materials and Methods

2.1 Culture of 3T3-L1 pre-adipocytes

For all experiments, 3T3-L1 adipocytes (ATCC) were cultured from frozen/thawed cells according to the protocol provided (American Type Culture Collection, 2011) in 12-well flat-bottom cell culture plates (BD Falcon) containing 2 mL of liquid medium per well. Cells were incubated in a humidified incubator with 5% carbon dioxide (CO₂) at 37°C.

Four distinct cell populations, grouped according to differentiation status and MeHg exposure time, were used in these experiments. A visual depiction of the names and characteristics of these populations is presented in Figure 2.1.

For those populations undergoing differentiation (populations “1” and “3” in Figure 2.1), cultures were grown to confluence using Growth Medium (see Table 2.1), followed by incubation with Growth Medium for two further days past confluence to initiate contact inhibition. At this point, cells were switched to Differentiation Medium containing IBMX, dexamethasone, and insulin. Incubation with Differentiation Medium continued for two days, with medium changed every 24 hours. Cells were then switched to medium containing insulin for two days, with medium again changed every 24 hours.

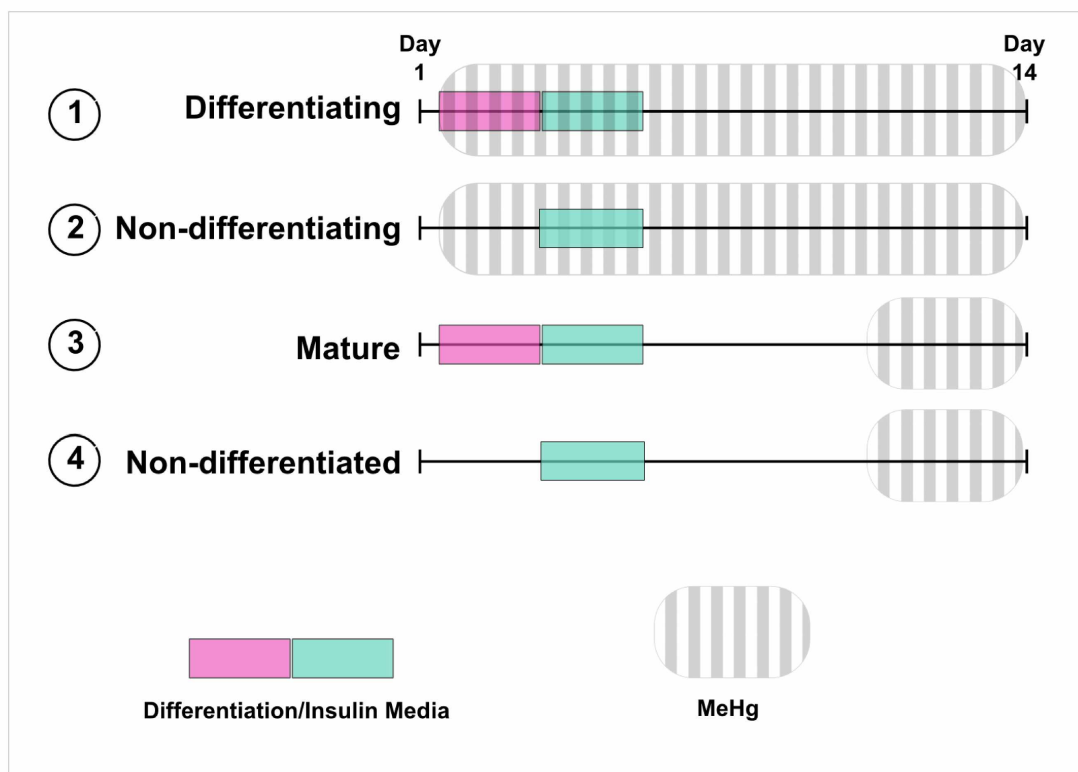


Figure 2.1: 3T3-L1 cell populations/MeHg exposure schemes. Populations 1 and 3 were given Differentiation Medium. Populations 3 and 4 were only exposed to MeHg at the conclusion of their growth and/or maturation periods.

Finally, cells were incubated with Maintenance Medium (with medium changed every 24 hours) for 10 days to maturity. Cell populations not undergoing differentiation (populations “2” and “4” in Figure 2.1) were grown to confluence similarly to the differentiating populations, but were given Growth Medium for Days 2 and 3 rather than Differentiation Medium. These populations were then given Insulin Medium and Maintenance Medium alongside the differentiating cells in the following days. Components of the different media described are summarized in Table 2.1, while the overall differentiation protocol is summarized in simplified form in Table 2.2.

Table 2.1: Media and chemical components for 3T3-L1 cell culture

Growth Medium	Dulbecco's Modified Eagle Medium (DMEM), high glucose (Sigma-Aldrich) 10% calf serum (Atlanta Biologicals) 3.7 g/L sodium bicarbonate (NaHCO_3) (Sigma Aldrich) 1% (by volume) penicillin/streptomycin (Gibco) 1% GlutaMax (Gibco) pH 7.4
Differentiation Medium	DMEM, high glucose 10% fetal bovine serum (FBS) (Atlanta Biologicals) 3.7 g/L NaHCO_3 1% (by volume) penicillin/streptomycin 1% GlutaMax 0.5 mM 1-methyl-3-isobutylxanthine (IBMX) (Sigma-Aldrich) 0.25 μM dexamethasone (Sigma-Aldrich) 2 $\mu\text{g/mL}$ insulin (Sigma-Aldrich) pH 7.4
Insulin Medium	DMEM, high glucose 10% FBS 3.7 g/L NaHCO_3 1% (by volume) penicillin/streptomycin 1% GlutaMax 2 $\mu\text{g/mL}$ insulin pH 7.4
Maintenance Medium	DMEM, high glucose 10% FBS 3.7 g/L NaHCO_3 1% (by volume) penicillin/streptomycin 1% GlutaMax pH 7.4

Table 2.2: 3T3-L1 cell differentiation protocol

Day Name	Procedure/Medium Used
	Grow cells to confluence in Growth Medium ; replace medium every 2-3 days
	Cells are confluent
	Add Growth Medium ; incubate 24 hours
Day 1	Change Growth Medium ; incubate 24 hours
Day 2	Add Differentiation Medium ; incubate 24 hours
Day 3	Change Differentiation Medium , incubate 24 hours
Day 4	Add Insulin Medium ; incubate 24 hours
Day 5	Change Insulin Medium , incubate 24 hours
Day 6 - 20	Add Maintenance Medium ; incubate and change every 24 hours for 8-15 days until cells are mature

Cells were viewed under an AMD EVOS Core digital inverted light microscope, and images were captured using the microscope software beginning after the first 24-hour incubation in Growth Medium (Day 1). Cells were imaged immediately *prior* to changing cell media; because of this, “Day 1” images represent the appearance of the cells after 24 hours of incubation with Growth Medium, “Day 2” images show cells after 24 hours in Differentiation Medium, and so on.

Upon each cell medium replacement, 1 mL of old media was collected from each well, saved in a 1.5 mL Eppendorf tube, labeled according to the scheme above (“Day 1” for media collected after 24 hours in Growth Medium, etc.) and stored at -20°C until analysis.

Methylmercury (MeHg) stock solution was obtained at 1000 ppm in H₂O (Alfa Aesar), and 1.4 µL were added to a 1.4 mL aliquot of medium to create a MeHg/medium solution of 1 ppm (1000 ng/mL). Appropriate amounts of this solution were added to each culture plate well to obtain the desired concentration in 2 mL of medium. Fresh MeHg/medium solution was made each day immediately prior to addition to cells, and solutions were discarded as hazardous waste after use.

Concentrations of MeHg in the media used were designed to approximate total mercury at levels that would be considered slightly elevated (25 ng/mL), elevated (100 ng/mL), and very elevated (200 ng/mL) in human blood (Hamade, 2014). A list of concentrations used, as well as MeHg/medium volumes required to reach each concentration in the well plate, are listed in Table 3. Individual cell cultures were either treated from Day 1 (during differentiation) or for several days after reaching maturity (post-differentiation).

Table 2.3: Volumes of MeHg added to cell culture media

Final [MeHg] (ng/mL)	1000 ng/mL MeHg solution added per well (μL)	Medium added per well (mL)
0	0	2.000
25	50	1.950
100	200	1.800
200	400	1.600

Appropriate personal protective equipment was used at all times when working with MeHg solutions. Precautions included the use of Silver Shield gloves, along with an additional outer nitrile glove, whenever concentrated MeHg stocks were opened, as well as lab coats and a chemical safety hood. All mercury-containing liquid or solid wastes, including cell media, Eppendorf tubes, and pipet tips, were disposed of as hazardous waste according to local regulations.

2.2 LDH assay

The lactate dehydrogenase assay is a colorimetric analytical assay used to quantitatively measure toxicity to cells in culture, particularly toxicity mediated by

compounds. The assay makes use of the enzyme lactate dehydrogenase (LDH), which under normal conditions is present within the cytoplasm of many different cell types, and is not normally secreted or released outside the plasma membrane of the cell. If a cell is damaged due to toxicity, the plasma membrane will be compromised, and LDH will be released into the culture media. The presence of LDH activity within cell media can therefore be used to determine cytotoxicity of a given compound or treatment to a population of cells based on quantification of LDH released from those cells.

The LDH assay has become a common means of assaying cytotoxicity, and so is generally performed using a commercially available kit. The LDH kit used in these experiments was the Pierce LDH Cytotoxicity Assay, which provides its own protocol, described briefly below.

Cell media were collected from 12-well cell culture plates as described in Section 2.1 above. Media samples were thawed at room temperature (RT) and centrifuged at 2000 rpm for 5 minutes to precipitate any cell debris. 50 μ L of cell medium supernatant were added to wells of a 96-well clear flat-bottom plate (BD Falcon) in duplicate. LDH positive control from the kit was added to 1 well, and cell-free differentiation media to another well as a blank. Each well then received 50 μ L of LDH reaction mix containing assay buffer and LDH substrate, a molecule that reacts with active LDH enzyme to form a red-colored product. Samples were incubated with reaction mix for 30 minutes at RT in the dark, and absorbance was read at 490nm in a BioTek Synergy HT plate reader using Gen5 software.

2.3 VEGF ELISA

The enzyme-linked immunosorbent assay (ELISA) is a sensitive quantitative assay used to determine concentrations of particular proteins within a mixture or biological sample. The assay is based on the principle that antibodies bind with high specificity to a particular antigen. ELISA is widely used both for research and for clinical diagnostics, and exists in several different forms, the most common of which is known as the sandwich ELISA (Figure 2.3).

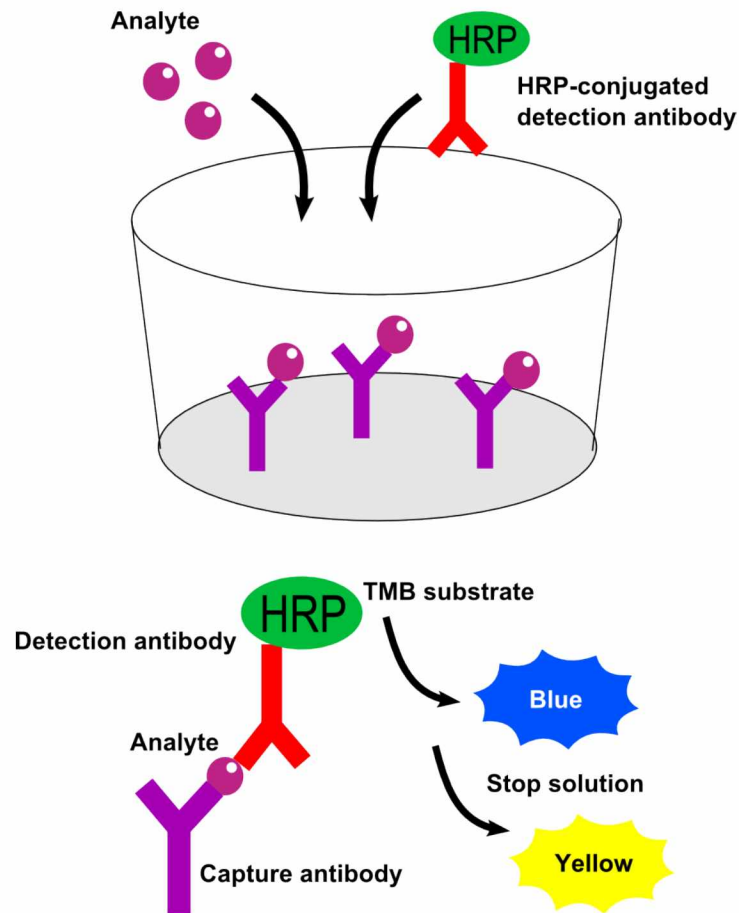


Figure 2.2: Schematic of generic sandwich ELISA (adapted from technical sheet provided by R&D Systems, www.rndsystems.com)

In a sandwich ELISA, microplate wells are coated with a capture antibody specific to the analyte of interest. Samples are added to the wells, and any analyte of interest present within the samples will bind with high affinity to the plate-immobilized antibody. After incubation with sample, a solution containing enzyme-conjugated secondary antibody also specific to the analyte of interest is added to the wells. The enzyme conjugated to this antibody produces a blue color upon incubation with a substrate solution, and after stopping the enzymatic reaction, a yellow color is generated. The plate can then be analyzed using a spectrophotometric plate reader in order to determine color density within each well; higher absorbance indicates a higher antigen concentration in the well. Each assay is generally run along with a standard curve containing known quantities of the analyte of interest, such that absorbance values of the unknown samples can be interpolated into the standard curve and used to determine antigen concentrations.

For these experiments, the analyte of interest was VEGF; the kit was the Quantikine Mouse VEGF ELISA kit produced by R&D Systems. The kit provides its own protocol, which is described briefly below.

Cell media were collected from 12-well cell culture plates as described in Section 2.1 above. Media samples were thawed at RT and centrifuged at 2000 rpm for 5 minutes to precipitate any cell debris. Cell medium supernatants from each MeHg condition were added to wells of the anti-VEGF coated microplate provided with the ELISA kit. A VEGF standard curve was created using VEGF standard supplied with the kit in a concentration range of 0-2000 pg/mL; media samples were diluted 1/2 prior to their addition to the plate in order to generate values that would fall within this standard curve. The plate was incubated for 2 hours at room temperature on a plate shaker, then rinsed five times using

wash buffer. Antibody conjugate was then added to each well and the plate was incubated for 2 hours at room temperature on a plate shaker. Wells were again washed five times with wash buffer. Color reagent was added, and the plate was incubated for 30 minutes at room temperature in the dark without shaking. Stop solution was added to quench the color reaction, generating a yellow color; absorbance was read at 450 nm in a BioTek Synergy HT plate reader using Gen5 software.

2.4 Statistical Analysis

All experiments were run in duplicate using two identically treated cell populations in separate plates as replicates. Media samples were collected from each plate every 24 hours, thus each "Day" time point had $n=2$ for each cell population/treatment condition. All statistical analyses and plots were prepared using GraphPad Prism software.

For statistical analyses of effects on differentiating cells, cell populations were grouped according to stage of differentiation: Differentiation (Days 2 and 3, when differentiating cells were grown in Differentiation Medium), Insulin (Days 4 and 5, when differentiating cells were grown in Insulin Medium), Maintenance (Days 6-8) and Maturity (Days 10-12). Measurements of VEGF/LDH for each stage were calculated as an average of measurements from each plate for the included days. Results were then plotted against MeHg concentration on an XY plot for each stage (8 points per plot) and a linear regression was performed. A trendline with a slope significantly different from zero ($p < 0.05$) was considered to represent a significant effect on LDH or VEGF with MeHg concentration.

Cell populations that did not undergo differentiation, or that were exposed to MeHg only at the end of the differentiation process (see Figure 2.1), were instead analyzed as a

single group for each population, rather than dividing into groups based on differentiation stage. Analyses comparing two groups of data (e.g. exposure with MeHg vs. no exposure) used a Student's t-test, with resulting p values less than 0.05 considered significant.

Chapter 3: Results

3.1 Morphological changes to 3T3-L1 cells upon differentiation and MeHg exposure

Observation of morphological changes in 3T3-L1 adipocytes in culture revealed that morphological changes are dependent on several different factors, including cell differentiation status, concentration of MeHg exposure, and time of MeHg exposure. Results for each condition are detailed below.

3.1.1 Changes to cells during differentiation

Differentiation of 3T3-L1 preadipocytes into mature adipocytes, as described by the protocol in Table 2.2, results in changes in cell morphology that are easily visible when viewing cells under a light microscope. Figure 3.1 shows untreated healthy confluent cells immediately prior to initiation of differentiation, i.e. immediately prior to addition of Differentiation Medium for the first time (Day 1; 3.1A), and mature cells after the differentiation process was complete (Day 14; 3.1B). Cells in 3.1B are notably more rounded and contain visible lipid vesicles indicative of successful differentiation.

3.1.2 Changes to cells after exposure to MeHg during differentiation

Addition of MeHg to cell culture media over a period of several days resulted in additional significant visible changes to morphology of the cells, not attributable simply to

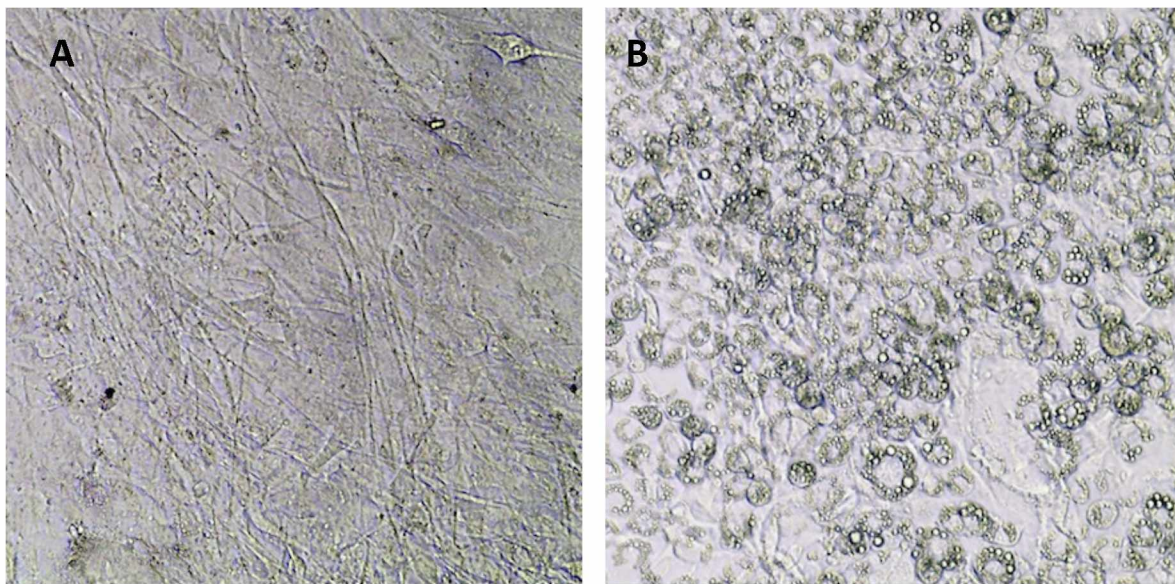


Figure 3.1: Healthy confluent 3T3-L1 cells. Cells are shown before (A) and after (B) differentiation.

processes of cell differentiation, that were likely indicative of cellular stress and/or toxicity. Such changes included a decrease in cell size and reduction in confluence (Figure 3.2J; 3.2L). These changes only occurred at MeHg concentrations at the higher end of the tested range (100, 200 ng/mL) and the incubation time required before seeing changes was positively related to MeHg concentration.

Figure 3.2 shows a comparison of cells during the first seven days of the differentiation process, both unexposed cells (0 MeHg) and cells exposed at the highest MeHg concentration (200 ng/mL MeHg). Images shown were taken at 24-hour intervals immediately before changing cell media.

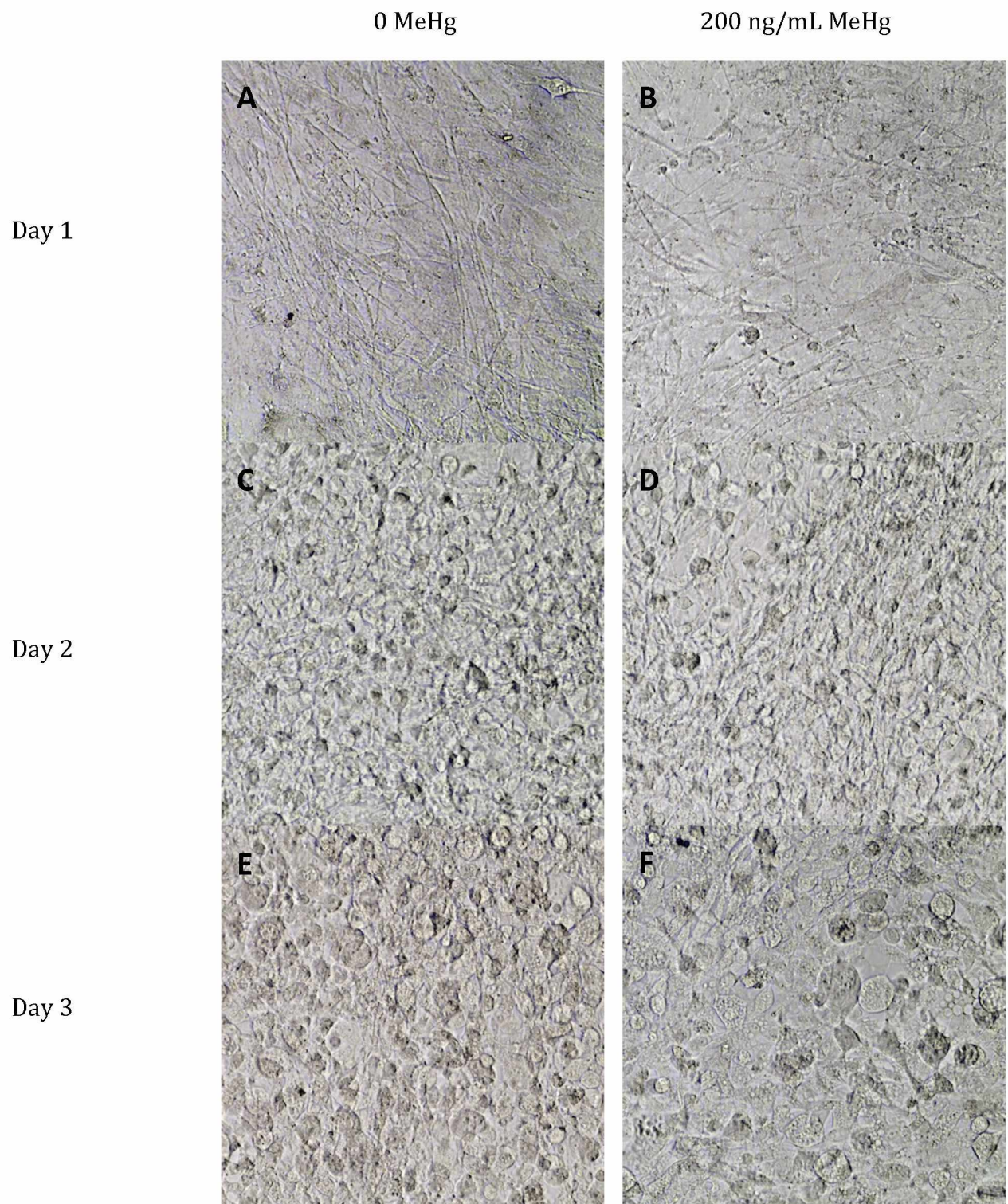


Figure 3.2: Differentiating 3T3-L1 cells exposed to 200 ng/mL MeHg. MeHg exposure causes visible changes to cell morphology.

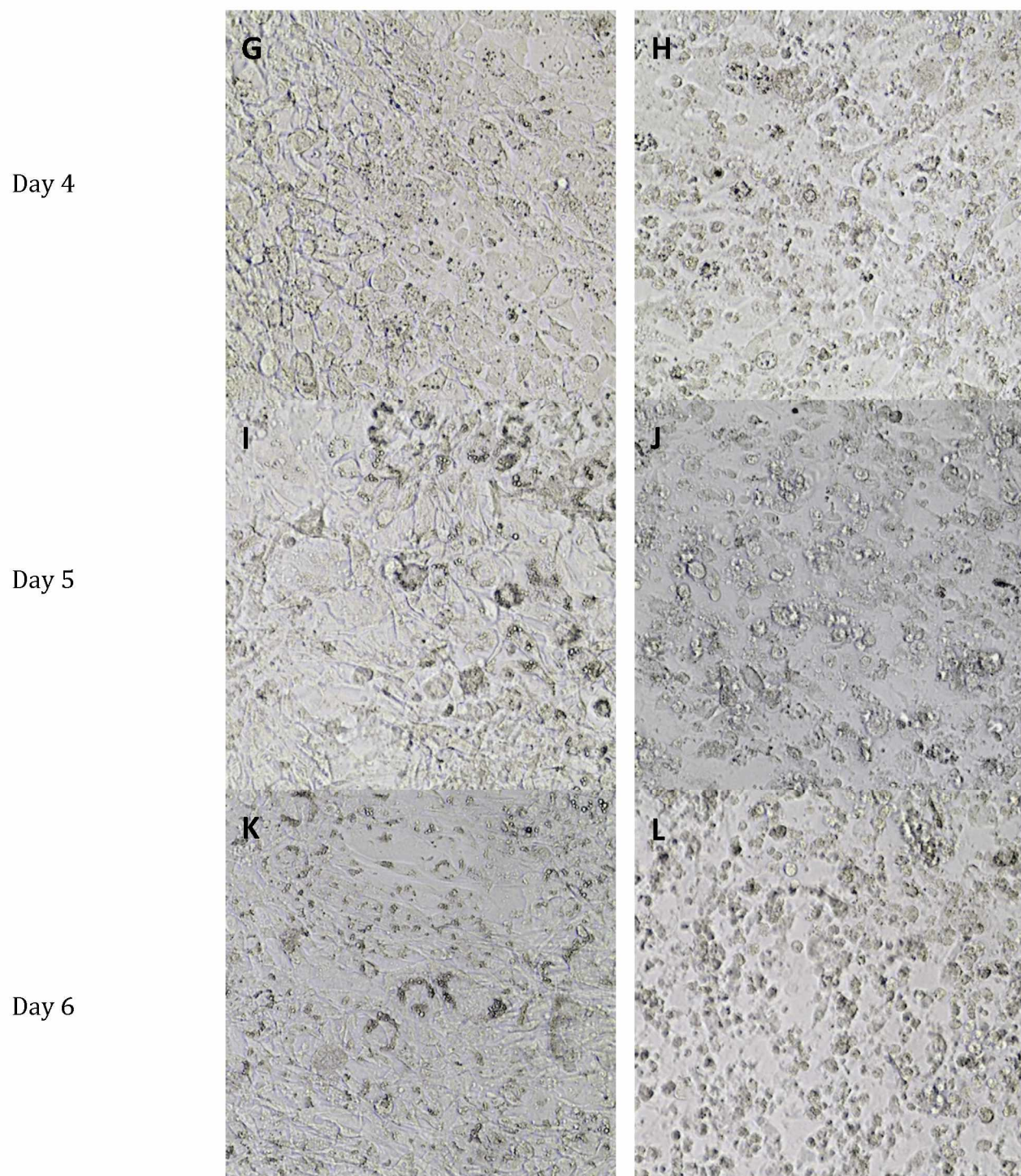


Figure 3.2 (continued): Differentiating 3T3-L1 cells exposed to 200 ng/mL MeHg.
MeHg exposure causes visible changes to cell morphology.

At Day 8 of exposure to 200 ng/mL MeHg, the cells were found clumped together and detached from the culture dish (not shown) indicating that the toxicity of MeHg had led to complete cell death through apoptosis.

A similar pattern of morphology change and cell death was seen in the cells exposed to 100 ng/mL MeHg, though cell detachment did not occur until Day 10 of exposure. Figure 3.3 shows a comparison of unexposed cells and 100 ng/mL MeHg-exposed cells at Day 10 after initiation of differentiation. The unexposed cells clearly show developing lipid vesicles, while the 100 ng/mL MeHg cells are shown detaching from the surface of the culture plate; a few developing lipid vesicles are nonetheless visible in these cells as well, indicating that processes of differentiation and cell morphology changes associated with the differentiation process may still be occurring to some extent in spite of cytotoxicity.

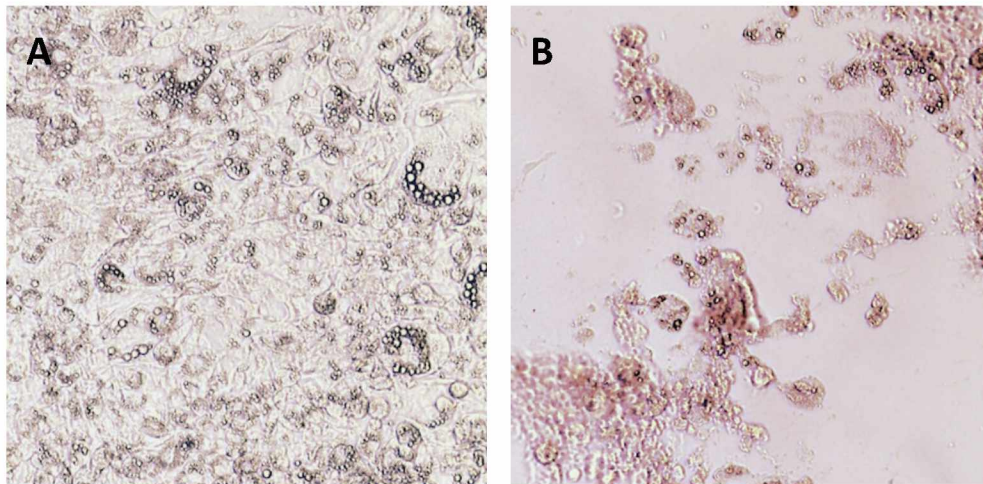


Figure 3.3: Differentiating 3T3-L1 cells exposed to 100 ng/mL MeHg. Unexposed cells (A) are shown compared to exposed cells (B) at Day 10.

Cells exposed to 25 ng/mL MeHg throughout differentiation showed no noticeable difference in morphology to exposed cells at Day 14, with no visible decrease in cell size or detachment from the culture plate (Figure 3.4). Qualitative observation of the number of lipid vesicles present suggests fewer in the exposed cells (Figure 3.4B), which may be due to natural variation in vesicle number and will need to be further evaluated quantitatively to determine if a true effect of MeHg on lipid storage is occurring. Nonetheless, the lack of overall morphological changes to the cells suggests that cytotoxicity of 25 ng/mL MeHg is less than that of 100 ng/mL or 200 ng/mL MeHg at 14 days of exposure.

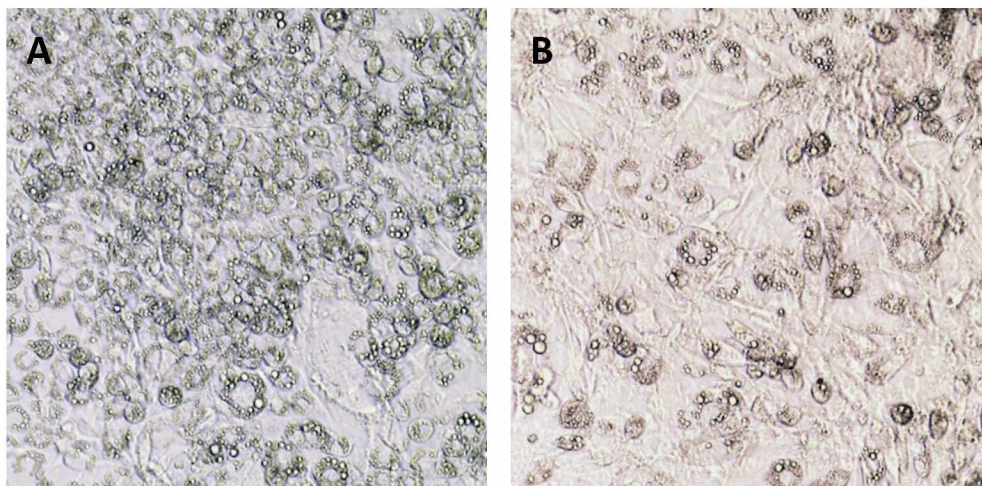


Figure 3.4: Differentiating 3T3-L1 cells exposed to 25 ng/mL MeHg. Unexposed cells (A; identical to 3.1B) are shown compared to 25 ng/mL MeHg-exposed cells (B) at Day 14

3.1.3 Changes to cells after exposure to MeHg without differentiation

An additional subset of cells were either exposed to no MeHg or to the highest concentration of 200 ng/mL, and were not differentiated: media used for these cells were

Growth Medium for Days 1-6, and Maintenance Medium for all subsequent days. Thus these cells were not exposed to IBMX/dexamethasone and did not experience the significant change in morphology associated with differentiation. Cells unexposed to MeHg did not experience any noticeable change in morphology over the course of the 14-day exposure; they maintained the morphology associated with undifferentiated pre-adipocytes (Figure 3.5A). Cells exposed to 200 ng/mL MeHg experienced morphological changes similar to those seen in the differentiating adipocytes exposed the same MeHg conditions, although complete detachment from the culture plate took approximately 1 day longer to occur (Figure 3.5B).

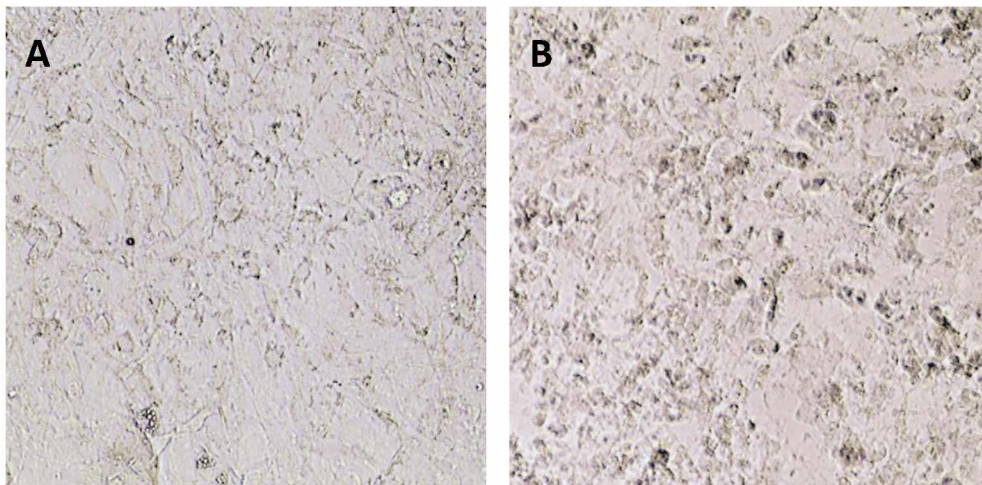


Figure 3.5: Non-differentiating 3T3-L1 cells exposed to 200 ng/mL MeHg. Unexposed cells (A) are shown compared to 200 ng/mL MeHg-exposed cells (B) at Day 7

3.1.4 Changes to cells after exposure to MeHg post-differentiation

Additional cell populations were differentiated and allowed to reach near-maturity without any exposure to MeHg, at which point they were then exposed to 0, 25, 100 or 200

ng/mL MeHg in order to determine effects of MeHg exposure on already-differentiated adipocytes. Figure 3.6 compares morphology of unexposed, differentiated adipocytes at Day 14 to those exposed to 200 ng/mL MeHg for 3 days beginning at Day 12; no significant differences in morphology are seen after exposure.

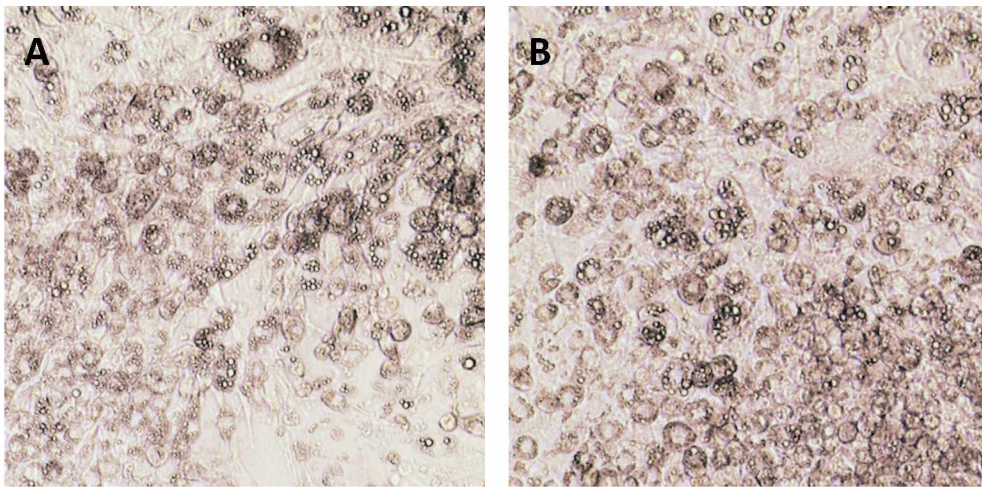


Figure 3.6: Mature 3T3-L1 adipocytes exposed to 200 ng/mL MeHg. Unexposed cells (A) are shown compared to 200 ng/mL MeHg-exposed mature adipocytes (B) at 3 days of MeHg exposure

3.2 Cytotoxic effects of MeHg exposure on 3T3-L1 cells

Quantification of cytotoxicity using the LDH assay revealed that toxicity of MeHg exposure on adipocytes is dependent on several different factors, including concentration of exposure, time of exposure, and cell differentiation status. Results for each condition are detailed below.

3.2.1 Cytotoxic effects on pre-adipocytes during differentiation

As detailed in Chapter 2, differentiating pre-adipocytes were exposed to different MeHg concentrations beginning at confluence (Figure 2.1, condition 1), and cytotoxicity was determined using the LDH assay performed on media collected every 24 hours of exposure during each stage of differentiation. Samples from the Differentiation stage were collected on Days 2 and 3, the Insulin stage on Days 4 and 5, and the Maintenance Stage at Days 6 and 8, with $n=2$ for each day. Cytotoxicity was quantified using absorbance of each sample at 490 nm following the LDH assay, a measurement which represents LDH activity and presence in the cell media. Results are shown in Figure 3.7.

The results show a significant increase in LDH activity, indicating increased cytotoxicity, with MeHg concentration during the Insulin phase (Days 4-5) as determined by a significant non-zero slope of the linear regression line when MeHg concentration is plotted against LDH activity (slope: 0.000586; y-intercept: 0.200; $p < 0.0001$). Cytotoxicity decreases in the 200 ng/mL population during the Maintenance stage; given the morphology of the 200 ng/mL cell population around Day 5-6 (see Figure 3.2J; Figure 3.2L) this decrease likely corresponds to the majority of the cells' contents having been already released into the medium during the process of programmed cell death. Because of this, the 200 ng/mL population was not included in the linear regression for the Maintenance stage. The 100 ng/mL remains elevated at this stage, and there is again a significant increase in cytotoxicity with MeHg concentration, as determined by a significant non-zero slope of the linear regression line when MeHg concentration is plotted against LDH activity (slope: 0.000594; y-intercept: 0.229; $p = 0.0016$). These results suggest that that an exposure

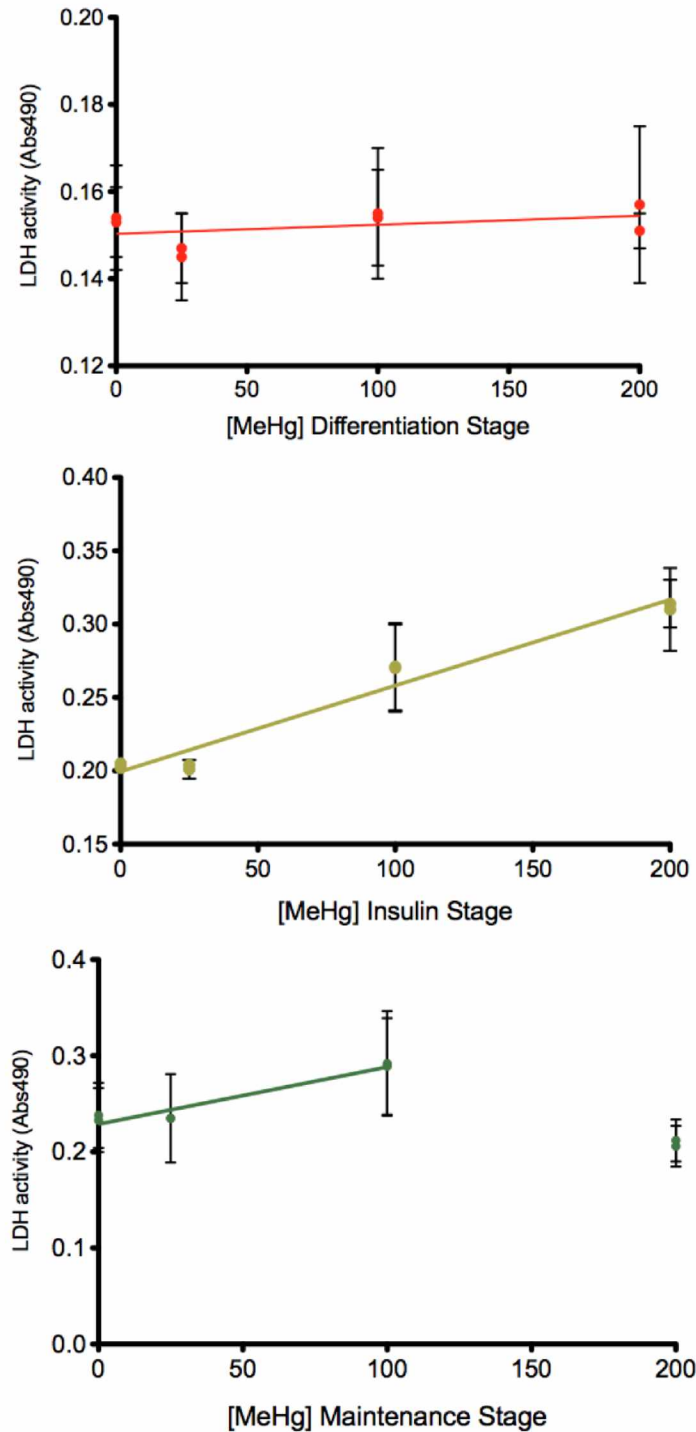


Figure 3.7: Cytotoxicity of MeHg in differentiating 3T3-L1 cells. Cytotoxicity is expressed as LDH activity as determined by LDH assay. A linear regression producing a significantly non-zero slope was considered significant ($p < 0.05$, Insulin & Maintenance stages). Each point represents $n=2$ (average of Day 2 and Day 3 for each sample); 200 ng/mL MeHg was not included in Maintenance plot (bottom) due to fewer viable cells left in culture during this stage.

concentration of 100 ng/mL requires a longer exposure time than 200 ng/mL in order to cause maximum cytotoxic effects.

3.2.2 Cytotoxic effects on non-differentiating pre-adipocytes

Two separate populations of cells, either unexposed to MeHg or exposed to 200 ng/mL MeHg, were not exposed to Differentiation Medium and thus retained their pre-adipocyte phenotype (Figure 2.1, condition 2). Media were collected from these cells every 24 hours over 14 days; the LDH assay was performed on media from Day 3 to Day 5 (n=4) to determine total cytotoxicity. Results are shown in Figure 3.8.

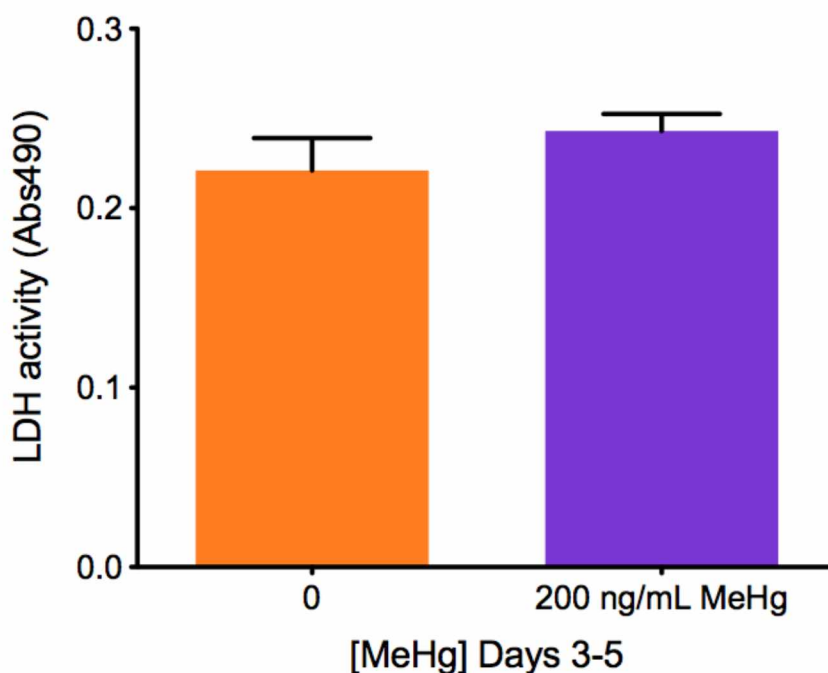


Figure 3.8: Cytotoxicity of MeHg in non-differentiating 3T3-L1 cells. Cytotoxicity is expressed as LDH activity as determined by LDH assay. Days 3-5 represent the time period during which significant cytotoxicity was seen in differentiating cells (see Figure 3.7).

Results show no significant difference ($p = 0.526$) in cytotoxicity between unexposed and MeHg-exposed cells between Days 3 and 5 of exposure, a time period in which significant differences in cytotoxicity were seen between unexposed and MeHg-exposed cells undergoing differentiation (Figure 3.7, Insulin Stage). This indicates that cytotoxicity of MeHg is different in 3T3-L1 cells depending on the cells' differentiation status.

3.2.3 Cytotoxic effects on mature adipocytes

Adipocytes that had been allowed to differentiate to-maturity while unexposed to MeHg during differentiation were either unexposed or exposed to 25, 100, or 200 ng/mL MeHg for three days starting at Day 12 after initiation of differentiation (Figure 2.1, condition 3). Media were collected every 24 hours, and results for the LDH assay for all samples within the three-day time period are shown in Figure 3.9 ($n=2$ for each point plotted). The slope of the resulting trendline is not significantly different from zero ($p > 0.05$), thus no significant relationship exists between cytotoxicity and MeHg exposure concentration when cells are exposed for 3 days after reaching maturity.

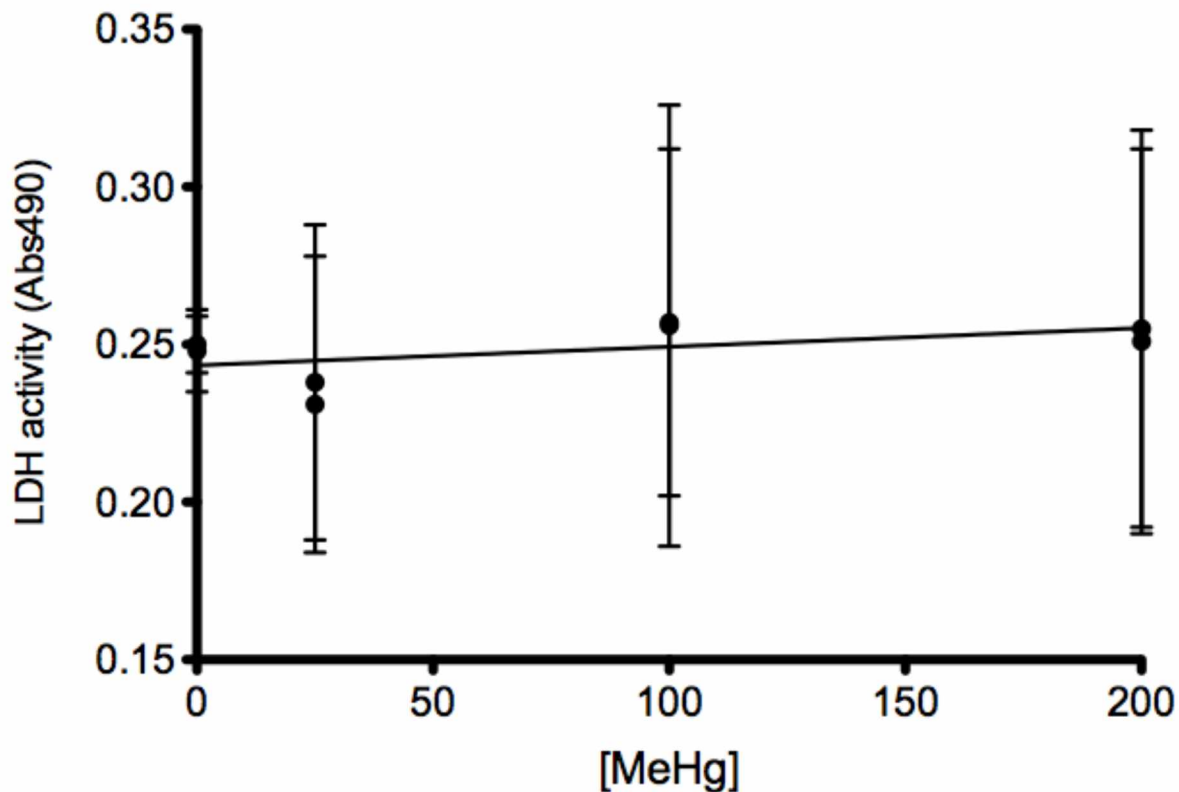


Figure 3.9: Cytotoxicity of MeHg in mature 3T3-L1 adipocytes. Cells were exposed to MeHg for 3 days after differentiation. Cytotoxicity is expressed as LDH activity as determined by LDH assay. Linear regression produced a slope not significantly different from zero ($p > 0.05$) and was considered not significant. Each point represents $n=3$ (average of Days 1-3 for each replicate)

3.2.4 Cytotoxic effects on non-differentiated cells after 12 days of no exposure

Cells that were both undifferentiated and unexposed to MeHg for 12 days were exposed either to no MeHg or to 200 ng/mL MeHg for three days to assess cytotoxicity, with $n=6$ for each exposure condition (Figure 2.1, condition 4). Results showed no significant difference in cytotoxicity between unexposed and MeHg-exposed cells for samples collected within the three-day exposure period ($p = 0.364$).

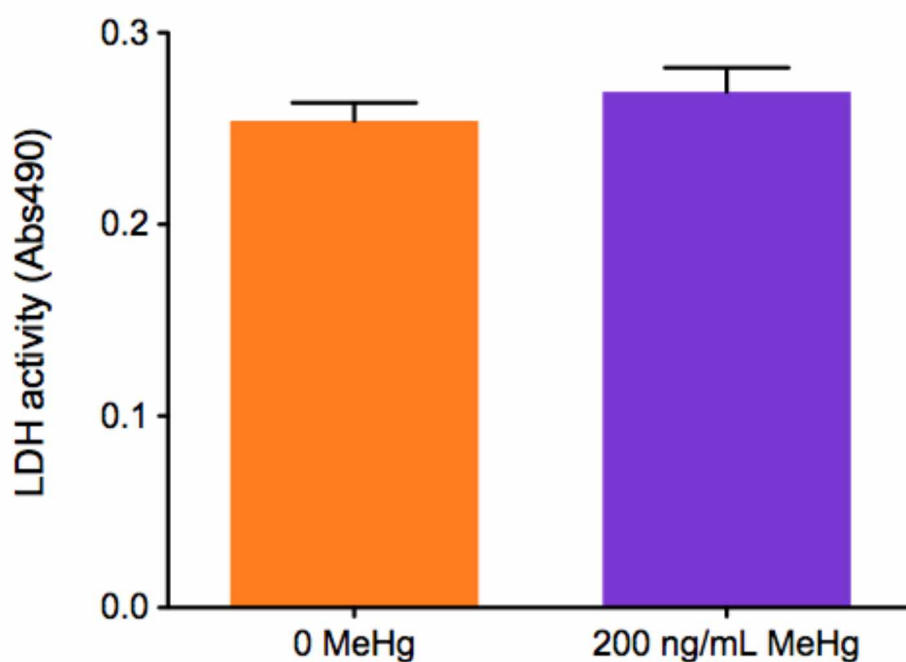


Figure 3.10: Cytotoxicity in non-differentiated cells exposed to MeHg for 3 days.
Cytotoxicity is expressed as LDH activity as determined by LDH assay.

3.3 Effects of MeHg exposure on VEGF secretion from 3T3-L1 cells

Quantification of VEGF in cell media established that, as in the case of cytotoxicity, secretion of VEGF by cells exposed to MeHg is dependent on concentration of MeHg, time of exposure, as well as cell maturity and differentiation status.

3.3.1 Effects on VEGF secretion from pre-adipocytes during differentiation

As detailed in Chapter 2, differentiating pre-adipocytes were exposed to different MeHg concentrations beginning at confluence (Figure 2.1, condition 1), and VEGF secretion was determined using a VEGF ELISA performed on media collected after each period of 24

hours of exposure during each stage of differentiation. Samples from the Differentiation stage were collected on Days 2 and 3, the Insulin stage on Days 4 and 5, the Maintenance Stage at Days 6 and 8, and the Maturity stage at Days 10 and 12, with n=2 for each individual day. Concentration results from this assay represent VEGF concentrations in 2 mL of cell media produced by a confluent layer of adipocytes in each well of a 12-well plate.

Results are shown in Figure 3.11 and indicate elevated VEGF secretion in all cells (exposed and unexposed) during the Differentiation stage as compared to other stages.

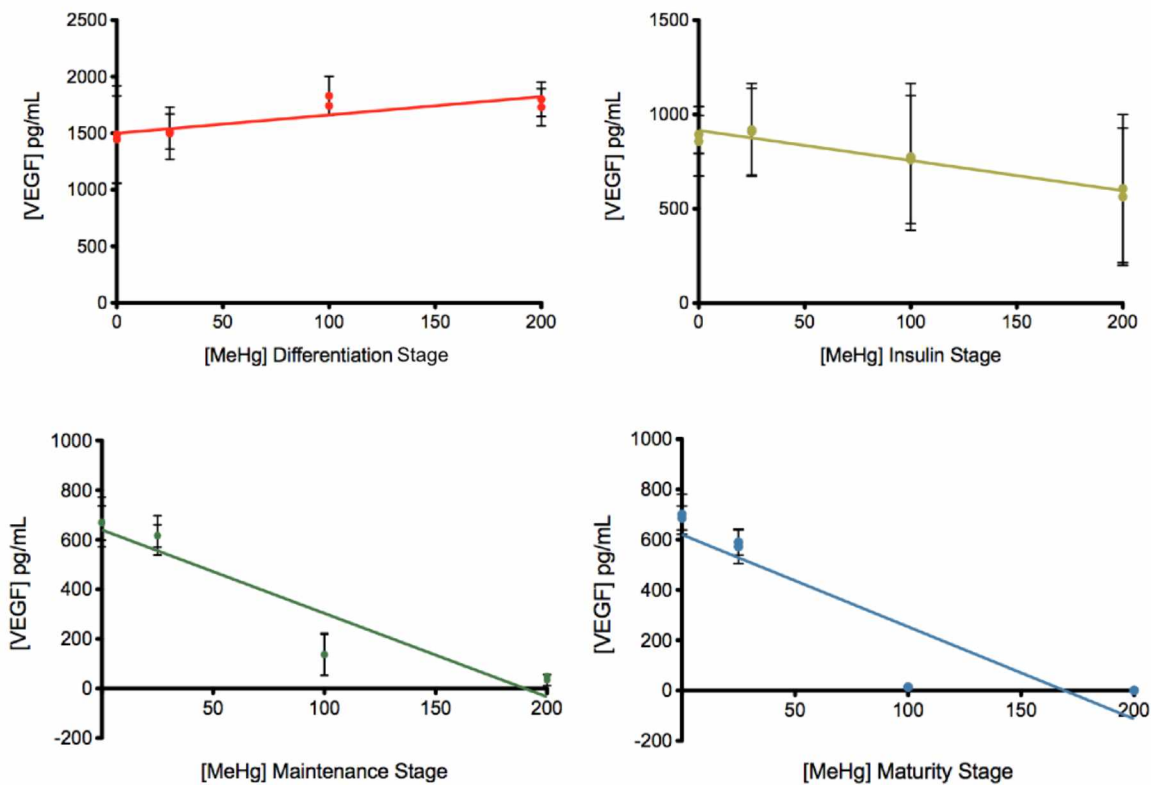


Figure 3.11: Effects of MeHg on VEGF secretion from differentiating cells. A linear regression producing a significantly non-zero slope was considered significant ($p < 0.05$, all stages). Each point represents n=2 (average of Day 2 and Day 3 for each sample)

This is likely associated with cells' exposure to IBMX and dexamethasone rather than any function of MeHg concentration (see Discussion).

During the Differentiation stage, a significant increase in VEGF secretion with MeHg concentration is observed, while a significant decrease with MeHg is seen from the Insulin stage onward. During the Maintenance and Maturity stages, VEGF secretion at the higher MeHg concentrations (100 and 200 ng/mL) decreases below those concentrations found in unexposed and 25 ng/mL-exposed cells; this is likely due to increased cytotoxicity of MeHg at higher concentrations leading to fewer viable cells being present rather than any specific mechanism of MeHg causing a decrease in VEGF secretion.

3.3.2 Effects on VEGF secretion from non-differentiating pre-adipocytes

Given that exposure to IBMX/dexamethasone in Differentiation Medium causes elevated VEGF secretion even without exposure to MeHg (Figure 3.11), we also quantified VEGF secretion from the non-differentiating cells (unexposed to IBMX/dexamethasone; condition 2 in Figure 2.1) in order to determine whether MeHg itself had any effect on VEGF secretion. Two separate populations of cells, either unexposed to MeHg or exposed to 200 ng/mL MeHg, were not exposed to Differentiation Medium and thus retained their pre-adipocyte phenotype (see Figure 3.5). Media were collected from these cells every 24 hours over 14 days; the VEGF ELISA was performed on media samples from Days 1 and 4 to quantify VEGF secretion (Figure 3.12).

Results from the undifferentiated cells (n=4 for each exposure group) showed no significant difference in VEGF secretion to unexposed cells between Days 1 and 4 ($p =$

0.152), indicating that MeHg itself does not cause an increase in VEGF secretion independently of the increase caused by Differentiation Medium.

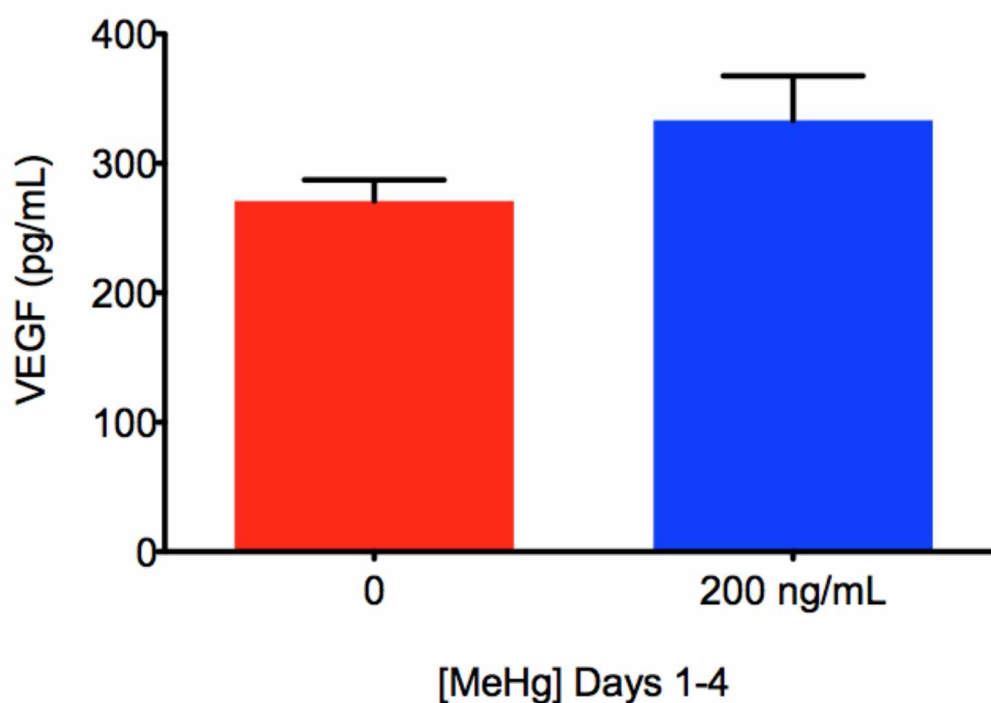


Figure 3.12: Effects of MeHg on VEGF secretion from non-differentiating cells

3.3.3 Effects on VEGF secretion from mature adipocytes

Cells that had been allowed to differentiate to-maturity while unexposed to MeHg during differentiation were either unexposed or exposed to 25, 100, or 200 ng/mL MeHg for three days starting at Day 12 after initiation of differentiation (Figure 2.1, condition 3). Media were collected every 24 hours, samples from the three-day period were all analyzed as a group (n=2 for each exposure concentration each day; 8 points total) and results for the VEGF ELISA are shown in Figure 3.13.

Linear regression of the plotted results revealed a significantly positive non-zero slope (slope: 0.944, y-intercept: 457; $p < 0.0001$), indicating a significant increase in VEGF secretion with increased MeHg concentration in mature cells. These results, in conjunction with the different effects in VEGF secretion seen in the Differentiation and Insulin stages of differentiating cells (Figure 3.11), indicate that effects of MeHg exposure on 3T3-L1 cells is dependent on concentration of MeHg and on differentiation status of the cells themselves.

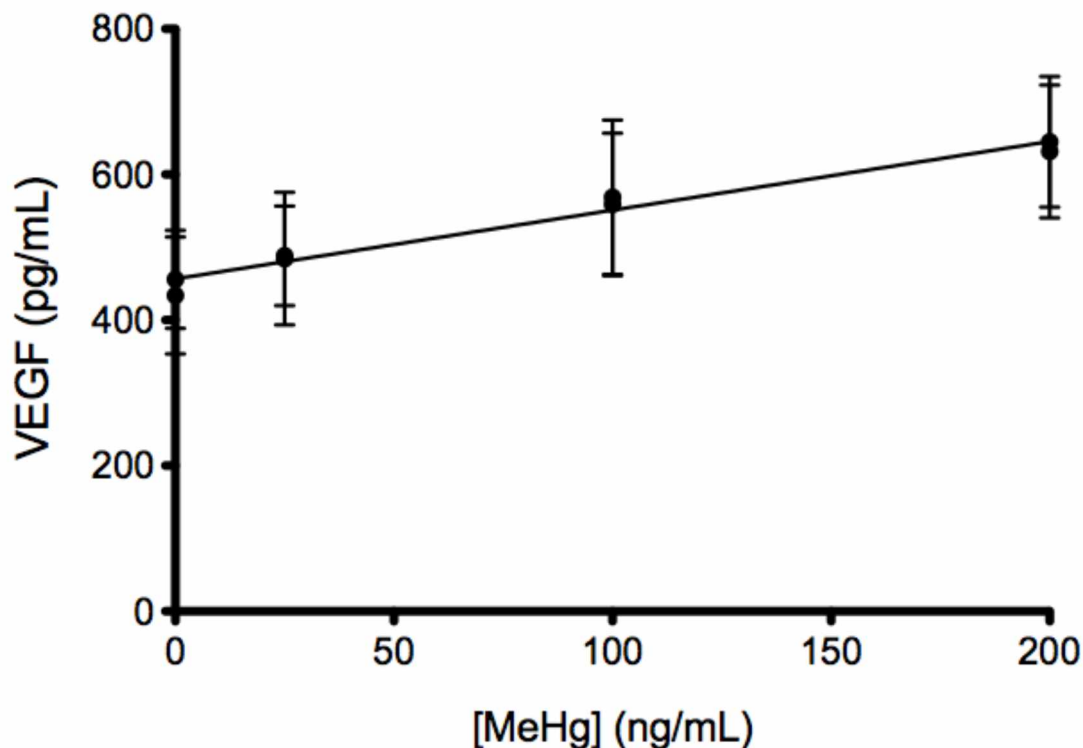


Figure 3.13: Effects of 3 days of MeHg exposure on VEGF secretion from mature adipocytes. Linear regression produced a significantly non-zero slope and was considered significant. Each point represents $n=3$ (average of Days 1-3 for each replicate)

3.3.4 Effects on VEGF secretion from non-differentiated cells after 14 days of no exposure

Cells that were both undifferentiated and unexposed to MeHg for 12 days were exposed either to no MeHg or to 200 ng/mL MeHg for three days to assess VEGF secretion (Figure 2.1, condition 4). Results showed no significant difference between exposed and unexposed cells ($p = 0.874$), further confirming that effects of MeHg on VEGF secretion are dependent on the differentiation status and/or maturity of the adipocytes at the time they are exposed to MeHg.

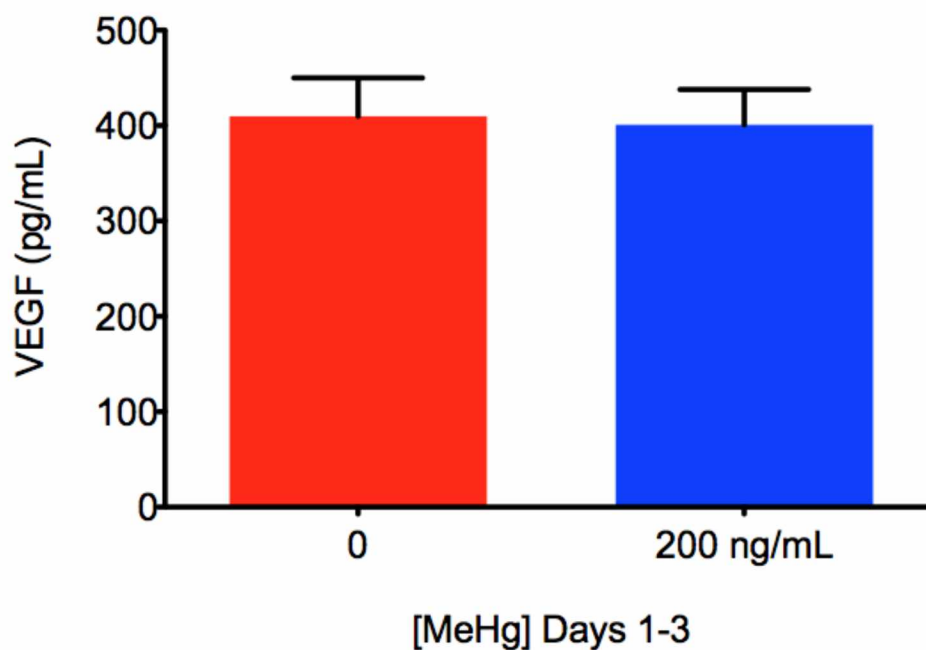


Figure 3.14: Effects of 3 days of MeHg exposure on VEGF secretion from non-differentiated cells. Cells were exposed to 200 ng/mL MeHg after a 12-day unexposed period

Chapter 4: Discussion and Future Directions

4.1 Discussion

4.1.1 Significance of overall results

Many of the laboratory-based studies published thus far on the health effects of MeHg exposure have focused on detrimental effects to the central nervous system, often with an emphasis on exposure to the developing fetus, as these are considered the critical target organ and most susceptible subpopulation, respectively (Hamade, 2014). In general, these studies have examined effects occurring at relatively high exposure levels not typical in most human populations. However, people who consume large amounts of MeHg-containing fish on a regular basis (e.g. Native populations and subsistence hunters in the Arctic) are regularly exposed to low levels of MeHg for longer periods of time than these studies represent. Though the nervous system is still considered the critical target organ, elevated levels of blood MeHg could potentially cause toxic effects on other tissues and cell types as well, including adipose tissue. Though several epidemiological and animal studies have addressed this possibility, few *in vitro* studies up to now have explored the details of such toxicity in depth. Our results, presented in Chapter 3, represent the first known examination of the effects of MeHg exposure to adipocytes *in vitro*, as well as one of only a few published experiments showing the cytotoxic effects of longer-term (>48 hours), low-level MeHg exposure (<200 ng/mL) on cells of any type in culture.

4.1.2 Concentration-dependent cytotoxicity of MeHg

Our results showed that the cytotoxic effects of MeHg on differentiating adipocytes are dependent on MeHg concentration as well as on stage of differentiation, with higher MeHg concentration producing maximum cytotoxic effects in earlier differentiation stages than lower concentrations. LDH activity, and thus cytotoxicity, was shown to be positively correlated with MeHg concentration, indicating that increased MeHg exposure causes increased cytotoxicity. This effect appeared most prominently during the Insulin stage of differentiation at Days 4 and 5, with cytotoxicity measurements decreasing for the highest exposure concentration (200 ng/mL) in the following stages, likely due to fewer viable cells present in culture rather than a physiologically significant decrease in cytotoxicity with time. Morphological examination of these cells shows the majority of the population undergoing cell death and detaching from the culture dish by Day 9 (for 200 ng/mL exposure) and Day 11 (for 100 ng/mL exposure).

Many cell culture studies, including several of those cited examining MeHg, assess cytotoxicity after a much shorter exposure time, often 48 hours/2 days. Our research shows that such experiments would have missed the cytotoxic effects of exposure to concentrations between 100-200 ng/mL, whose effects are not apparent until Day 4 and later.

Our experiments examined effects on cytotoxicity with increasing MeHg exposure concentration and showed a significant relationship. However, it is unknown whether this relationship would remain significant if low concentrations (25 ng/mL and lower) were used exclusively, given that morphological examination of cells exposed to 25 ng/mL MeHg did not reveal the same changes in phenotype as the cells exposed to higher concentrations.

Even longer exposure to these lower concentrations may indeed lead to toxic effects; as stated above, future studies of MeHg toxicity in cell culture should account for time of exposure in determining whether or not cytotoxic effects are occurring.

The toxicity of 200 ng/mL MeHg exposure was not limited to adipocytes undergoing differentiation; indeed, comparison of the morphological changes in non-differentiating vs. differentiating adipocytes exposed to 200 ng/mL MeHg saw similar reduction in cell size and detachment from the culture plate, though observation of these effects appeared to require a slightly longer (~1 day) incubation time with the same MeHg concentration in the undifferentiated cells as compared to the differentiating ones. The LDH assay performed on non-differentiating cells did not show an increase in cytotoxicity around Day 5 as had been seen in the differentiating cells, but it is unknown whether cytotoxicity may have increased/peaked at a later time, which would confirm a differentiation-dependent effect on cytotoxicity. Further experiments will need to be performed in order to determine this effect with certainty, with LDH assay measurements taken every day for both non-differentiating and for differentiating cells, and resulting cytotoxicity plotted over time. If future results show that a longer MeHg exposure time is in fact required for toxicity in non-differentiating cells, one explanation could be that the process of differentiation itself exerts some chemical and physical stresses on the cells that lessen their ability to tolerate the toxic effects of MeHg exposure.

4.1.3 Concentration-dependent effects of MeHg on secretion of VEGF

Our results also represent the first known study examining effects of MeHg exposure, at any level and for any duration, on the secretion of VEGF from adipocytes.

VEGF, while long known as a target in development of cancer therapies, has only in recent years been recognized for its possible role in metabolic dysfunctions such as diabetes and obesity. While its precise role in the development of these disorders has not yet been fully elucidated, studies have shown that VEGF may significantly affect metabolic status of an organism when overexpressed or absent in adipose tissue (Elias et al., 2013; Sun et al., 2012). Because of this, compounds that affect secretion of VEGF from adipose tissue may represent possible avenues for research into the mechanisms of metabolic disease.

Exposure to MeHg during adipocyte differentiation had varying effects on VEGF secretion depending on the MeHg concentration used and the duration of exposure. All cells, regardless of MeHg exposure, experienced elevated VEGF secretion during the Differentiation stage, during which they were grown in Differentiation Medium. This elevation is likely due to the effects of the compounds in the Differentiation Medium itself, namely IBMX and dexamethasone; the former is known to increase production of cAMP (see Chapter 2) and exposure to IBMX has been shown to increase VEGF secretion even in healthy cells (Gregoire et al., 1998). For this reason, cell populations were evaluated for VEGF secretion beginning at Day 2 (the Differentiation stage) so confounding effects of exposure to Differentiation Medium could be discounted.

It is worth noting that non-differentiating cells (cells not exposed to IBMX/dexamethasone) did not experience a significant increase in VEGF secretion upon treatment with MeHg (Figure 3.12). This could either confirm that MeHg exposure has no significant effect on VEGF secretion in either differentiating or non-differentiating cells, or could indicate that any effects on VEGF secretion are only occurring in differentiating cells and are more difficult to visualize due to effects of components of Differentiation Medium

on VEGF secretion. Further experiments assessing VEGF secretion after each individual day of MeHg exposure would be useful in determining what effects, if any, MeHg has on VEGF secretion from differentiating cells.

In later differentiation stages (after the Insulin stage), VEGF secretion decreases significantly with increased MeHg concentration, with higher exposure concentrations of 100 and 200 ng/mL showing VEGF secretion of nearly zero. This effect is presumably due to cytotoxicity and cell death rather than a specific effect of MeHg, an effect that becomes especially obvious once the cells have physically detached from the culture plate and thus are no longer present in the medium at all.

Although VEGF secretion showed a significant increase with MeHg concentration in the Differentiation stage of differentiating cells and in mature adipocytes, it remains to be seen whether this effect is primarily driven by increases at the higher levels of MeHg exposure (100 and 200 ng/mL), and whether exposure to 25 ng/mL MeHg in itself produces a significant effect or not. Further replicate experiments will need to be performed at this exposure level at a similar exposure duration in order to determine whether changes in VEGF secretion are a physiological effect due to MeHg or whether they fall within a normal range of variation. Because this level of exposure was not associated with significant morphological changes to the cells as described previously in this chapter, a decrease in VEGF secretion could represent a potentially interesting sub-lethal effect to the cells that would be worth exploring experimentally in further detail.

4.1.4 Physiological relevance of results

As mentioned previously, the central nervous system is considered the target organ of MeHg toxicity in humans, though MeHg at low levels in blood may also be capable of producing physiological effects in a variety of tissues, including adipose tissue. Our studies used levels of MeHg in cell media meant to represent concentrations that would realistically be found in blood, either consistently or transiently, of humans consuming MeHg-containing fish. Our results showed that these MeHg concentrations are capable of causing changes in the structure and function of adipose cells, possibly providing preliminary evidence that MeHg could cause physiological effects in organs other than the brain and nervous system.

While the effects of MeHg on cultured adipose cells as observed in this study were significant, particularly with regard to cytotoxic effects, their relevance with regard to MeHg exposure in a whole organism remains to be determined. Cell lines in culture provide a simple and effective model for understanding molecular effects of toxic compounds, but as is the case with MeHg, such compounds are generally not consumed in isolation, and are also often metabolized or biotransformed to some extent before they reach their target organs or tissues. Direct exposure of cells to MeHg, as in our experiments, may therefore only be a first step in understanding specific toxic effects of MeHg *in vivo*. Exposure to MeHg in humans generally occurs through consumption of fish, so many physiological and digestive processes must necessarily occur before MeHg from the fish even becomes available to interact with human tissues. Because of this, important interactions between MeHg and the digestive system of the human consumer, or between MeHg and other compounds contained within the fish itself, may be missed in cell culture studies. Further

experiments will likely need to be carried out (see 4.2.4 below for examples) in order to account for some of these possibly confounding factors.

4.2 Future Directions

4.2.1 Effects of MeHg exposure on secretion of other adipokines

Adipokine signaling within adipose tissue is known to be important in maintaining metabolic homeostasis; dysfunction in signaling has been shown to be associated with metabolic dysfunction and disease (see Introduction). In this study, we focused on secretion of one particular adipokine, VEGF, which has been an increased focus of research in recent years due to its possible role in type 2 diabetes and obesity. Many other adipokines, however, are known to have roles in the development of these diseases when over- or under-expressed in adipose tissue (Elias et al., 2013; Sun et al., 2012), so future studies may focus on some of these other molecules as well to determine whether MeHg affects their secretion from adipocytes.

Examples of adipokines that may be the focus of future MeHg/adipocyte studies include interleukin-6 (IL-6) and tumor necrosis factor (TNF). IL-6 is an inflammatory marker known to be secreted by adipocytes, and which can impair insulin signaling if overexpressed in adipose tissue (Murdolo & Smith, 2006). TNF (formerly known as TNF α) is another pro-inflammatory adipokine shown to be upregulated in adipose tissue in obesity (Trayhurn et al., 2005). Preliminary studies from our lab showed no measurable TNF in cell media in either MeHg-exposed cells or in unexposed cells, but, future

experiments could explore effects of MeHg exposure on the action of other compounds known to stimulate TNF.

4.2.2 Cytotoxic effects after brief MeHg exposure

Our experiments so far have focused on cytotoxic effects of chronic exposure of adipocytes to MeHg. Results showed significant toxicity to the cells at concentrations of 100 ng/mL and above over an exposure period of up to 14 days. It is currently unknown whether this extended exposure over a period of many days to weeks is required in order for the toxic effects of MeHg to manifest. Future experiments may expose cells to similar MeHg concentrations to those used in this study (up to 200 ng/mL) with exposure limited to a shorter period of 24-48 hours, followed by an extended period of no MeHg to determine whether such a brief exposure would be sufficient to lead to cytotoxicity. Such experiments would provide useful information toward determining the specific molecular mechanisms responsible for MeHg toxicity, such as whether a particular “threshold” of MeHg within a cell must be reached in order for toxic effects to occur, and whether extended incubation of cells with MeHg allows this threshold to be overcome.

4.2.3 Destination of MeHg within the cell

The precise molecular mechanisms for MeHg’s toxicity in cells have not yet been determined in detail, and such mechanisms are often difficult to elucidate due to signaling cross-talk and other confounding factors. It would be worthwhile, therefore, to first determine the ultimate destination of MeHg within a cell in order to possibly narrow down

its toxicity mechanisms. Numerous studies have investigated the subcellular fate of mercury compounds, including MeHg, in whole organisms (He & Wang, 2011; Farina et al., 2003; Massaro, 1999) but few thus far have examined fractions of cultured cells. Future experiments to this end could involve treatment of cells with MeHg known to cause cytotoxicity, as in the current study, followed by lysis and subsequent fractionation of cells, and mercury analysis to determine subcellular destination of mercury at various time points during exposure. Since MeHg preferentially binds to cysteine residues on proteins, further studies could potentially include total proteome analysis to examine specific protein targets that may be found enriched in MeHg in treated cell populations.

4.2.4 Confounding effects of omega-3 fatty acids on toxicity of MeHg

As described in Section 4.1.4 above, human exposure to MeHg often occurs through consumption of predatory fish, and thus other compounds present within the fish may affect the toxicity of MeHg to cells *in vivo*. Though evidence has existed for some time to show that omega-3 fatty acids found in fish may have a mitigating effect on MeHg toxicity (Hamade, 2014), only recently have studies been performed examining this phenomenon in cultured cells. Nøstbakken et al. (2012) exposed neuronal cells in culture to five concentrations of MeHg with and without supplementation of omega-3 fatty acids, and determined that certain fatty acids do in fact decrease the toxic effects of MeHg to the cells. Since MeHg exposure concentrations in this particular study were considerably higher than those used in our experiments, and exposure time was shorter, it would be worthwhile going forward to attempt a similar experiment setup using our adipocyte cell line and

exposure times/concentrations that we determined will produce toxic effects, and assess whether omega-3 fatty acids have any confounding effects on MeHg toxicity.

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